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A FILTER-PASSING VIRUS OBTAINED FROM DERMACENTOR ANDERSONI.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 17, 1926.)

In the course of a bacteriological study of *Dermacentor andersoni*, the wood tick concerned with the transmission of Rocky Mountain spotted fever[1] and of tularemia,[2] a filter-passing virus was met with and, apparently, carried through seven generations *in vitro*, as well as through experimental animals. The history of the tick material with which these studies were made is as follows:

Source of the Virus.

The tick from which the virus was isolated was one of a lot of 51 "drag" ticks collected in Saw Tooth Canyon, Montana, in the spring of 1925 and sent me through the courtesy of Dr. R. R. Parker, of the United States Public Health Service. Four ticks of this lot, after having been kept in the refrigerator (10–12°C.) for 54 days, were placed for 48 hours at 37°C. preparatory to tests for infection with spotted fever virus, according to the procedure of Spencer and Parker.[3] The tests were first made by allowing the ticks to feed for 72 hours, each on a normal guinea pig. Ticks 1, 3, and 4 became engorged with blood; Tick 2 was found dead. The guinea pig on which Tick 1 had fed developed typical spotted fever, the others remained well. The ticks were then eviscerated, the viscera were individually suspended in 4 cc. of salt solution, and 1 cc. of each suspension was injected into a normal guinea pig. The suspensions of Ticks 1 and 2 induced typical spotted fever, that of Tick 4 produced no reaction. The sus-

[1] Ricketts, H. T., *J. Infect. Dis.*, 1907, iv, 141.

[2] Francis, E., *J. Am. Med. Assn.*, 1925, lxxxiv, 1243.

[3] Spencer, R. R., and Parker, R. R., *Pub. Health Rep., U. S. P. H.*, 1924, xxxix, 3027.

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pension of Tick 3, which contained an unusually large number of microorganisms very similar to those found in infected ticks, induced a fever resembling spotted fever but one which conferred no immunity to the spotted fever virus. It is this infective agent existing in Tick 3 with which the present report is concerned.

Simultaneously with the injections into guinea pigs, each tick suspension was inoculated into special culture media, which are described in another paper,[4] and duplicate series of tubes were kept at 26°C. and at 37°C. The presence of ordinary bacterial contamination was

TABLE I.

Tick No.	Result of feeding.	Result of inoculation.
1	Typical spotted fever.	Suspension (containing moderate number of organisms similar to those in suspension of Tick 3) induced typical spotted fever.
2 (found dead).	— (Perhaps never attached.)	Suspension (containing some microorganisms) induced typical spotted fever.
3	— No immunity developed.	Suspension (containing unusually large numbers of microorganisms) induced fever very similar to that of spotted fever but none of the skin lesions of the disease. Immunity test, 17 days after inoculation, negative.
4	— No immunity developed.	Suspension (in which no microorganisms could be demonstrated) produced no reaction. No immunity developed.

controlled by the use of plain broth and agar slants. The culture made with the suspension of the dead tick, Tick 2, was discarded because of mold contamination. Cultures made from the suspensions of Ticks 1, 3, and 4 were tested on guinea pigs at various intervals, beginning as early as 72 hours of incubation. The amount of the culture used for inoculation was 0.1 cc. or less and was usually suspended for convenience in 1 to 2 cc. of saline.

No fever or other reaction developed in guinea pigs which had

[4] Noguchi, H., *J. Exp. Med.*, 1926, xliii, 515.

received the 72 hour cultures of Ticks 1 and 4. When tested with spotted fever virus 18 days after the inoculation, the animals inoculated with the Tick 4 cultures developed spotted fever, those which had received Tick 1 cultures proved immune. No real culture, however, was obtained with the suspension of Tick 1; it was completely inert when tested again 25 days later.

The cultures made with the suspension of Tick 3, however, gave rise to exactly the same type of fever in guinea pigs as had the original material. The 72 hour cultures proved active, and as time went on their activity gradually increased. As no microorganisms could be detected by dark-field examination or by any staining method, the infective agent present in the culture will be referred to here as a virus.

The virus is aerobic; it is capable of growing at 26°C. and 37°C. and has been continued in subcultures for seven generations at 37°C. It does not grow in ordinary broth or on slant agar. It produces a slight turbidity when grown in serum media containing certain carbohydrates (mixed sugars). No putrefactive odor or gas is produced. The blood and spleen,—the latter organ always much enlarged, dark red, and soft,—of guinea pigs responding to the cultures of the virus are infective, and continuous passages from animal to animal up to the 7th generation have been maintained with the material taken on the 3rd, 4th, and 5th days of fever. No microorganisms have been seen in cultures made with the blood or spleen of such animals. The virus was recovered from the blood of infected guinea pigs. It proved to be filterable through Berkefeld N filters.

Chart 1 shows the type of temperature curve observed in infection of guinea pigs with the virus under discussion. The result of an immunity test with spotted fever blood after recovery is also indicated in the chart.

EXPERIMENTAL.

Original Material.

B-C Tick 3, fed for 72 hours on guinea pig infected with spotted fever virus, Barlow-Cooper strain. 54 days later, fed on normal guinea pig 72 hours. *Result of feeding:* Animal showed no sign of infection. *Result of inoculation of suspended viscera:* Fever (Chart 1) developed 72 hours after inoculation; animal remained

febrile 7 days. Blood withdrawn at the height of fever sterile. No scrotal or ear lesions appeared, and subsequent inoculation with spotted fever virus (17 days after inoculation of tick suspension) induced the typical symptoms and lesions of the disease.

Culture Experiments.

1st generation.—A normal guinea pig received 0.1 cc. of culture (special semi-solid and slant media) kept 3 days at 26°C. after inoculation with 0.1 cc. of suspension of Tick 3. After 4 days the animal developed high temperature which continued for 4 days (105, 106, 104.5, 104.4°F.). No scrotal lesions appeared. Tested for immunity by injection of spotted fever virus (guinea pig blood) after 23 days. No protection.

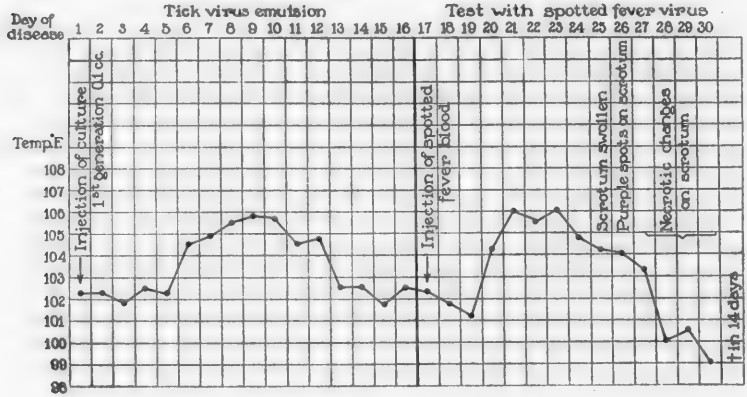

CHART 1.

Similar material, kept 3 days at 37°C., induced fever after 3 days incubation, which lasted 6 days (104.4, 105.6, 105, 103.8, 104.4, 104.2°F.). No scrotal lesions at any time. No immunity to spotted fever virus, as shown by immunity test 31 days after culture inoculation.

The same material, after 10 days at 37°C., induced a similar febrile reaction in two guinea pigs. In one, fever appeared after 4 days and lasted 6 days (104.6, 105.6, 105.6, 105.4, 105.2, 104.6°F.). No scrotal lesion developed. The animal showed no immunity against spotted fever when tested 12 days later. The second animal reacted with fever after 4 days and was killed for examination on the 5th day of fever (104.4, 105.2, 105.6, 106, 105°). The spleen was greatly enlarged and dark red, not unlike the spleen of a guinea pig infected with spotted fever; but no other lesions were found.

After 13 days at 37°C., the same material induced after 3 days incubation a

fever lasting 7 days. No scrotal lesions developed. When tested with spotted fever virus, 15 days after the inoculation of culture, this animal escaped infection. Two other guinea pigs which were inoculated with the same culture material and also reacted with fever did not prove to be immune to the spotted fever virus.

After 17 days at 37°C., the material was tested for its virulence by injecting guinea pigs with dilutions, as shown in Table II.

A second set of cultures was made with the same suspension after it had been kept 10 days at 0°C., and the culture tubes were placed at 37°C. for 8 days. A guinea pig which received 0.1 cc. of culture developed high temperature after 3 days incubation and remained continuously febrile for 8 days (104.6, 106.4, 106, 105.2, 105.6, 105, 104.8, 104.2°F.). No scrotal lesions developed at any time.

2nd generation.—Subcultures, made on the same media as the original cultures, and kept for 10 days at 37°C., were injected into two guinea pigs. High temperature developed in both animals after 6 days and was continuous in one for 6 days (104.4, 104.4, 104.6, 104.8, 104.4, 104°F.) and in the other for 5 days (105, 105.4,

TABLE II.

Amount injected.	Incubation period.	Duration of fever.	Immunity tests with spotted fever virus.
cc.	*days*	*days*	
1	3	7	No protection.
0.1	4	7	" "
0.1	3	8	Test not made.
0.01	4	7	No protection.
0.001	5	4 (105.4, 105.6, 104, 104.8°F.)	" "
0.0001	5	7 (Chart 2.)	Test not made.

105.2, 104.6, 104°). No scrotal lesions appeared. The first animal was tested for immunity to the virus 16 days after the culture inoculation. It proved immune. The second was tested with spotted fever virus after the same period. It reacted typically.

A second set of subcultures was kept 9 days at 37°C. and tested in normal guinea pigs. The animals had high temperature after 5 days which continued for 6 days (104, 104, 105.4, 105.8, 106, 104°F.). No scrotal lesions developed, and no protection against the spotted fever virus could be demonstrated. After 13 days at 37°C., the material gave rise after 3 days to high fever which lasted 7 days (104, 104, 105.5, 106.8, 107, 105.5, 104.5°), but no scrotal lesions appeared (Chart 3).

3rd generation.—Subcultures from tubes of the 2nd generation, kept 4 days at 37°C., induced in guinea pigs, after 6 days incubation, a fever lasting 5 days (104.5, 106.5, 106, 105.5, 105°F.) (Chart 4). There were no scrotal lesions. Another set of subcultures, after 8 days at 37°C., induced mild fever after 10 days incubation.

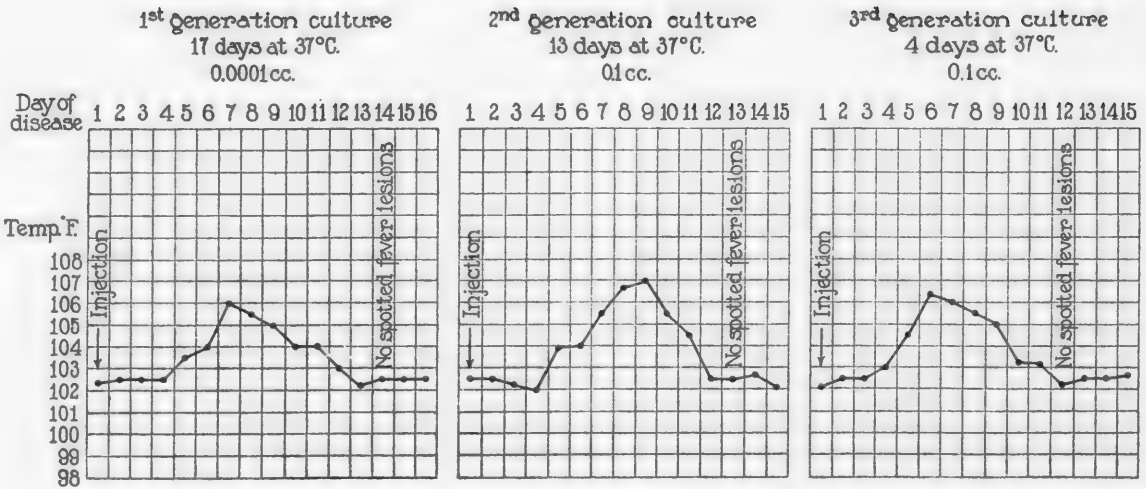

CHART 2. CHART 3. CHART 4.

In Charts 2, 3, and 4 are presented temperature curves of some of the guinea pigs inoculated with cultures of the 1st, 2nd, and 3rd generations.

4th generation cultures, 7 days old at 37°C., induced in one guinea pig after 10 days incubation a fever lasting 5 days, and in another fever of 3 days duration.

5th generation.—7 day old cultures, kept at 37°C., induced a febrile reaction lasting 4 days, the incubation period being 10 days.

6th generation cultures, 10 days old at 37°C., induced fever of 5 days duration after an incubation period of 7 days.

7th generation.—Subcultures from the 6th generation, after 4 days at 37°C., induced fever of 5 days duration following an incubation period of 10 days.

The number of positive reactions became less constant with further subcultures, the virus becoming gradually attenuated.

Macacus rhesus 1.—The monkey received an intraperitoneal injection of 1 cc. of mixed cultures of the virus, including one of the 1st generation (28 days old) and one of the 4th (8 days old). A fever fluctuating between 104°F. and 104.8° developed after 3 days and lasted 6 days. No spotted fever lesions appeared in the animal.

Guinea Pig Passages.

1st passage.—Guinea Pig XVIII-84B developed fever (104–105.5°F.) 5 days after receiving a 1st generation culture (13 days old at 37°C.), the temperatures recorded on successive days being 105.2, 105.4, 106, 105.5°F. The animal was killed on the 5th day of fever. The spleen was enlarged, and its appearance suggested that of the spleen in spotted fever infection. The blood, which was free from contaminating microorganisms, as tested by cultures, was injected intraperitoneally into two normal guinea pigs.

2nd passage.—Guinea Pig XVIII-121A[5] developed fever after 4 days which continued for 7 days (106.2, 105.4, 104.6, 104°F.). Guinea Pig XVIII-121B also showed high fever after 4 days (105.6, 106.4, 105.4, 105.4°F.). It was killed on the 4th day of fever for passage, cultural experiments, and virulence tests on monkeys, rabbits, and guinea pigs.

3rd passage.—Citrate blood from the animal just mentioned was injected into four guinea pigs, the first receiving 1 cc., the second 0.1 cc., the third 0.01 cc., and the fourth 1 cc. The fourth guinea pig received in addition 2 cc. of serum from rabbits immunized to spotted fever, of a titre such that 0.1 cc. neutralized 100 M.L.D. of spotted fever virus (guinea pig blood). The incubation period in each instance was 7 days, and the duration of fever was 2 to 3 days in the first three animals and 6 days in the fourth. There was no evidence of any neutralizing effect on the virus by spotted fever antiserum.

[5] Twenty-two ticks were placed on this animal on the 3rd day of fever and allowed to feed 5 days; nineteen became engorged, three were found dead.

The same blood was injected into *Macacus rhesus* 2 and a rabbit intraperitoneally, each receiving 2 cc., but no reaction followed in either instance.

After the 3rd passage, successive transfers were made as routine, two guinea pigs being used in each passage, with two sets of cultures set up for each, until the 8th passage, 3 months after the 1st, when the work was discontinued. No increase in virulence was noted, but on the contrary the duration of fever tended to become shorter and the incubation period longer. At each passage the blood was tested for ordinary bacterial invasion, as for example by bacilli of the paratyphoid group; no such invasion was discovered.

Filtration Experiments.

Cultures of the 1st, 2nd, and 3rd generations, grown on the special semisolid and slant media, were pooled and diluted with an equal volume of 0.9 per cent salt solution. The suspension, which contained no demonstrable microorganisms, was thoroughly shaken and passed through a Berkefeld filter N. The clear, light brownish filtrate was injected into four guinea pigs in amounts of 4, 3, 2, and 1 cc., respectively. The first three animals developed, after 4 days incubation, typical fever (104.5–105.5°F.) which was continuous for 7 days; the guinea pig which was inoculated with 1 cc. showed slight fever for 2 days. These animals were tested for immunity by injections of virulent spotted fever blood, but all had typical severe infections.

A guinea pig which had been injected as control with 1 cc. of the unfiltered suspension became ill in 4 days and passed through a course of fever lasting 5 days (106.2, 105.6, 106, 104.6, 104.6°F.).

Subcultures made with the filtrate yielded an active culture, which was subsequently tested for filterability, with positive results. Neither filtrate nor cultures contained any demonstrable microorganisms.

The question arose whether the infective agent revealed by the inoculation of guinea pigs had not merely survived in the culture tubes at 37°C. The original suspension, preserved at 3°C., was tested in guinea pigs in various dilutions on the 7th and 17th days after its preparation. The results, which are recorded in Table III, show that 1 cc. of the original material induced infection, but not 0.1 cc. or less. The culture of the 1st generation, on the other hand, when 17 days old was effective in a dose of 0.0001 cc.

As has already been mentioned, the original suspension contained an enormous number of minute, non-motile, Gram-negative microorganisms resembling spotted fever organisms. These did not grow on the media employed for cultivating the filterable virus under discussion.

Transmission of the Virus from Infected Guinea Pigs to Ticks and Vice Versa.

Experiment 1.—Seven ticks which had been fed for 5 days on a guinea pig in the febrile stage of the virus infection were placed separately on seven normal guinea pigs and allowed to feed for 5 days. In one of the animals fever developed after 8 days (104–105°F.) and lasted 4 days. The others escaped infection.

The tick which had induced infection was killed 119 days later and a portion of the emulsified viscera inoculated into a guinea pig. Fever (104.5–105.5°F.) developed after 48 hours and lasted 8 days. Death occurred on the 11th day. There were no lesions of spotted fever. The spleen was soft and considerably enlarged. No bacteria could be demonstrated in cultures made from the blood and spleen. Inoculation of blood withdrawn during the febrile stage induced the usual prolonged febrile, non-fatal reaction in guinea pigs; no spotted fever lesions developed.

TABLE III.

Amount of tick suspension.	Kept 7 days at 3°C.	Kept 17 days at 3°C.
cc.		
1	Usual fever.	Usual fever.
0.1	No reaction.	No reaction.
0.01	" "	" "
0.001	" "	" "

Smears of the tick revealed only a few microorganisms, but sections showed a large number of minute Gram-negative elements in the lumen of the intestine as well as in the viscera. Cultures made with the suspension yielded no growth.

Experiment 2.—A female tick, which had failed to induce infection by biting, survived 110 days and laid several lots of eggs. The tick and 40 of the eggs were separately suspended in saline, and a portion of each was injected into guinea pigs. No fever developed. In the smears and sections of the tick and eggs were found a number of Gram-negative microorganisms, and cultures yielded a growth of *B. rickettsiformis*.[4] The finding was of interest in showing hereditary transmission of this non-pathogenic microorganism, which had already been found in the ovaries and egg cells of ticks.

Experiment 3.—Four ticks of a lot of eleven which had been fed for 5 days on a guinea pig infected experimentally were still living after 124 days and were separately fed on guinea pigs, but no fever was induced. The remaining seven ticks were dead but only partly dried, and a single suspension in salt solution was made of their viscera. The guinea pig injected with a portion of this suspension developed after 8 days incubation a fever of 5 days duration. The blood withdrawn on the 4th day of fever contained no contaminating bacteria. The filtrate of the emulsion failed to induce infection.

This survival of the virus in ticks for several months at refrigerator temperature is not surprising, since it occurred also in culture. A guinea pig receiving 0.2 cc. of 1st generation culture of the virus, preserved at 2°C. for 5 months, developed the typical febrile reaction after 4 days and remained febrile for 6 days, when it was killed for transfer and culture. There was no bacterial invasion.

The foregoing experiments show that the virus is taken up by ticks from the blood of the infected guinea pig, but that transmission of the infective agent to other guinea pigs by the bite of infected ticks seldom occurs under the conditions described. The presence of the virus in ticks can be demonstrated more definitely by inoculation of guinea pigs with tick emulsions. That all ticks which are fed on infected guinea pigs do not take up the virus, however, is shown by the negative results in some instances.

Cross-Neutralization Experiments.

It has been repeatedly noted that guinea pigs which have responded to the virus under discussion do not acquire immunity against the spotted fever virus. Conversely, guinea pigs which have passed through the spotted fever infection were found to be quite as susceptible to this virus as normal animals. A potent spotted fever immune serum failed to neutralize the cultured virus.

SUMMARY.

An invisible, filter-passing virus, pathogenic for the guinea pig, and capable of cultivation on special media for at least seven generations, has been isolated from a tick of the species *Dermacentor andersoni*. One of two monkeys (*Macacus rhesus*) inoculated became infected, and in one rabbit the result was negative.

The virus has been transmitted from infected guinea pigs to ticks as also in one instance by tick feeding from an infected tick to a guinea pig The presence of the virus in the tick is more easily demonstrated by the inoculation of guinea pigs with a suspension of the tick viscera. Continuous high fever (104.5–106.5°F.) and enlargement of the spleen are the chief symptoms of the infection in guinea pigs. After the febrile attack the guinea pigs are not susceptible to reinoculation with the cultured virus but are subject to infection with the virus of spotted fever, and *vice versa*.

THE ORGANISM ASSOCIATED WITH SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS.

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PLATE 1.

(Received for publication, March 31, 1926.)

In a previous communication[1] we described a specific infectious cystitis and pyelonephritis of cows apparently not observed previously in this country. Owing to the inclusion of cases of other types of infections of the kidneys in the reports of certain writers and the slight comment on the bladder lesions, considerable confusion seems to have arisen both in regard to the organism and its method of reaching the kidney. The organism most frequently obtained in Europe thus far has failed in the hands of several observers to reproduce the disease.

Höflich[2] and Enderlen[3] cultivated from affected kidneys a Gram-positive diphtheroid. Enderlen named it *B. pyelonephritis boum.* Ernst,[4] who obtained pathologic kidneys from 24 cows slaughtered at the abattoir, found diphtheroids similar to those cultivated by Höflich and Enderlen in 11 instances. The others were associated with *B. pyogenes, B. coli,* and the tubercle bacillus. These workers agree that the diphtheroid is not pathogenic for the smaller animals. They obtained only negative results by intravenous injection of cultures into cows. Ernst first injected sterile sand into the bladder of a cow and on the following day 100 cc. of broth culture. Violent inflammation ensued but subsided after 9 days. This experiment, with the negative experiments reported by himself and others, led him to question the relationship of the diphtheroid to pyelonephritis.

In our first communication little was reported concerning the bacteriological findings. In all our cases we were able to readily cul-

[1] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1925, xlii, 593.
[2] Höflich, D., *Monatsch. prakt. Tierheilk.*, 1891, ii, 337.
[3] Enderlen, D., *Z. Tiermed.*, 1890–91, xvii, 325.
[4] Ernst, W., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 549, 660.

tivate a diphtheroid from the urinary sediment and from the pelvis of affected kidneys. As a rule the cortex was not invaded by the bacillus although in a few instances it was obtained from cortical lesions of advanced cases. The bacilli were usually found in practically pure culture, but at times cocci and streptococci were encountered in small numbers. From the morphology and lack of pathogenicity for small animals as noted by Enderlen, Höflich, and Ernst, it is evident that our cultures resemble those obtained by these workers. From 6 cases we cultivated the organism from the kidney. From the urine of 20 definitely affected cases the same organism was obtained. In the main it is true that the bacilli usually occur in the pelvis of the kidney in pure culture. They always predominate in the urine and frequently occur in pure culture, although at times cocci of various sorts are found. These bacteriological findings associated with lesions of uniform character led us to conclude that we were dealing with a specific infectious disease.

The Morphological and Cultural Characters of the Organism.

The bacillus is non-motile and in the urinary sediment is found in dense masses. The rods are slender and vary from 2 to 3μ in length. They stain well with the ordinary stains and are Gram-positive. Those from the tissues are not as pleomorphic as those from the earlier transfer cultures, although many may show polar granules or swollen ends. The morphology of cultures grown in broth is more variable; short coccoid forms, beaded rods with swollen ends, as well as the usual slender rods are abundant (Fig. 1). Occasionally a stalk with rudimentary branches may be observed.

The bacillus is readily grown on ordinary media. In liquids, such as broth or urine, aggregates of considerable size are found at the bottom of the tube. On the surface of agar or serum the growth is raised, grayish white, and dry. On potato the growth at first is usually grayish white, but later it becomes a dingy yellow and the potato turns brown. Blood serum and gelatin are not liquefied and hemolysin is not produced. In litmus milk the growth reaction is characteristic. The litmus at the bottom of the tube is reduced, here the casein coagulates, but the upper stratum is deep blue and coagulates slowly. The casein is digested slowly. Of the carbohydrates tested only dextrose has been attacked. No gas is produced, but a final hydrogen ion concentration of pH 4.9 to 5.0 is a regular finding. Lactose, saccharose, maltose, and mannitol are not attacked, since only alkali is produced in tubes containing them. These findings were constant for the 26 strains studied.

TABLE I.

The Agglutination Affinities of Various Strains of the Diphtheroid Organism.

Case No. and source of the strain.	Antiserum Strain 1132 (kidney), Herd A.						Antiserum Strain 1143 (kidney), Herd B.						Antiserum Strain 1151 (urine), Herd B.					
	Dilutions of serum.						Dilutions of serum.						Dilutions of serum.					
	1:20	1:50	1:100	1:200	1:500	1:1,000	1:20	1:50	1:100	1:200	1:500	1:1,000	1:20	1:50	1:100	1:200	1:500	1:1,000
1132. Kidney. Herd A.	C.*	C.	++++	+++	+++	−	C.	C.	C.	C.	++++	+	C.	C.	++++	++	−	−
273. Urine. Herd A.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	++	+	C.	C.	C.	++	+	+
139B. Urine. Herd A.	C.	C.	C.	+++	+	+	C.	C.	C.	C.	++	+	C.	C.	C.	++++	++	+
1143. Kidney. Herd B.	C.	C.	C.	++	+	−	C.	C.	C.	C.	+++	+	C.	++	+	+	+	−
1151. Urine. Herd B.	C.	C.	C.	C.	+	−	C.	C.	C.	C.	+	−	C.	C.	C.	++	+	−
1154. Urine. Herd B.	C.	C.	++++	+++	+	+	C.	C.	++++	++++	+	±	C.	C.	C.	++	−	−
1243. Urine. Herd B.	C.	C.	C.	+++	+	±	C.	C.	C.	C.	++	+	C.	C.	C.	+++	++	+
1223. Urine and kidney. Herd C.	C.	C.	++++	+	−	−	C.	C.	C.	++	+	−	C.	C.	C.	+	−	−
1225. Urine. Herd C.	++++	++++	++	+	+	−	++++	+++	++	+	+	−	++++	+++	+++	++	+	−
K. M. Urine. Herd D.	C.	C.	C.	+++	+	−	C.	C.	C.	+++	+	−	C.	C.	C.	+++	+	−
K. Urine. Herd E.	++++	++	++	+	+	−	+++	++	++	++	+	−	++	++	++	+	+	−

* The degree of agglutination has been recorded as follows: C., complete clearing of the fluid as result of agglutination; ++++, a heavy clumping without complete clearing; +++, well defined clumping; ++, less well defined clumping; +, a definite deposit on the bottom of the tube; ±, a slight deposit on the bottom of the tube.

Since it was possible to show that we were dealing with a group of organisms whose morphological and cultural characters were identical, it was desirable to know whether strains from different herds were immunologically related.

Our material was drawn from five herds in this vicinity and, since there was no exchange of cows between the herds, we were presumably dealing with fairly independent strains. To establish their immunological relationship, rabbits were immunized with cultures of the various strains. After the serum had reached a maximum agglutinin titer the animals were bled. The serum was tested against salt solution suspensions of all the cultures. The tendency of certain strains to clump spontaneously was largely overcome by rapid transfer on artificial media and mechanical shaking of the suspension with glass beads. Inasmuch as the 26 strains reacted to about the same degree, the details concerning only a few from each herd are given in Table I.

In the main it can be said that all cultures reacted to a greater or less degree to three agglutinins prepared by immunizing rabbits with single strains. It is of further interest to note that those isolated from the kidneys at autopsy or from the urine of cows in Herds A, B, C, and D, behaved in an almost identical manner to all agglutinins. The strain from Herd E also agglutinates with the specific sera but to a less degree.

Pathogenicity for Animals.

Our cultures, like those described by the European workers, are not pathogenic for rabbits or guinea pigs. Rabbits easily withstand an intraperitoneal inoculation with 10 cc. of a 48 hour bouillon culture. Guinea pigs and white mice are equally resistant. We were unable to show that the organism produces a soluble toxin. Young guinea pigs injected intraperitoneally with filtrates of unheated 48 hour and 5 day broth cultures fail to react. In our first communication mention was made of experiments in which broth cultures filtered through Berkefeld candles were injected into and beneath the skin and instilled on the conjunctiva of infected cows. In no instance were allergic reactions obtained.

A number of experiments were made with cows. In two instances cows were injected intravenously with 5 cc. and 10 cc. of freshly isolated broth cultures. Slight temperature reactions occurred shortly

after the injections, but the organism never appeared in the urine. At autopsy the urinary system failed to show abnormalities. On two occasions sterile swabs moistened with cultures were introduced into the vagina. The organism failed to develop in the vagina, and as far as we could determine the bladder never became invaded. The findings at autopsy were negative. In two instances cows were fed large quantities of freshly isolated broth cultures. One developed typical cystitis associated with the diphtheroid. The other remained normal. The experiment was repeated with entirely negative results. The feces were examined a number of times after the cows had been fed the cultures but the organism could not be found.

Instillation of small quantities of culture into the bladder led to definite infection. Since this is the first recorded experiment in which infection with pure culture of the diphtheroid has been established the details are given.

Cow 1100.—A Holstein that had been under observation at the Institute for over 10 months. Samples of urine were obtained for examination on several occasions before the injection. The urine was normal and contained only a few cocci. On Jan. 24, 1925, the bladder was emptied by means of a catheter and 3 cc. of a 24 hour broth culture in the 3rd generation was introduced. On Jan. 26, the urine contained a little albumen; the sediment contained a few red blood cells, leucocytes, cocci, and diphtheroids. The next day the bacilli were much more numerous, but the urine presented no gross abnormalities. On Jan. 28, the sample was slightly turbid. It reacted to HNO_3. The sediment contained epithelial cells, leucocytes, and bacilli in enormous numbers. Plate cultures contained the organism in practically pure culture. On this day the cow strained considerably at the close of urination. Daily examinations were made until Feb. 4. On this day the urine was dark brown in color. It reacted markedly with HNO_3 and gave an Esbach reading of $\frac{1}{4}$ per cent albumen. There was considerable sediment composed of red cells, leucocytes, and large numbers of bacilli. For the next 3 days red cells were always observed in the sediment. From this time until Mar. 10 the urine was examined daily. It always reacted to tests for albumen. The amount and character of the sediment varied. At times red cells made up the bulk of it, at others leucocytes predominated. The bacilli were always present in large numbers (Fig. 2). On Mar. 11, 1925, the cow was slaughtered at an abattoir. The kidneys and ureters presented no gross abnormalities. The bladder, however, showed characteristic lesions (Fig. 3). The mucosa of the ventral half was swollen. It was thrown up into broad raised folds. The color of the involved portion was yellowish pink and mottled with scattered, well defined, irregular, deeply reddened areas. Under slight mag-

nification the reddened areas were composed of points, lines, and patches of deep red extravasations located within the mucous membrane. The bacilli were readily cultivated from the mucosa overlying the congested areas. Microscopic examination of fixed and stained sections from the kidney and ureter failed to show abnormalities. Those from the bladder revealed definite lesions. The mucosa was degenerated. The nuclei were swollen and stained faintly; many were observed to lie within a clear vacuole. In certain instances the nuclei were shrunken, granular, and stained deeply. Beneath the mucosa there was a band of fibrinous exudate containing many round cells and a few leucocytes. The submucous connective tissue was edematous. Aggregates of round cells were scattered throughout the submucosa; they were particularly numerous just beneath the mucosa. The edema extended into the muscular coat.

Another cow was inoculated into the urethra with 5 cc. of broth culture. Albumen appeared in the urine 3 days after injection and the bacilli were present in the sediment in practically pure culture. The cow was under observation for over 3 months. The urine was examined frequently. The bacilli were always present. Albumen could usually be detected; leucocytes and red cells were usually present in the sediment and at times in considerable quantities. The cow was slaughtered 100 days after the inoculation. The kidneys and ureters failed to show gross changes. About five-eighths of the mucosa of the bladder was swollen. It was thrown up into broad folds and rounded hummocks. The involved portions varied from yellow to orange in color and were sprinkled with red areas varying from points barely visible to hemorrhages 1.5 to 2 cm. in diameter. Microscopic examination of fixed and stained sections (Fig. 4) revealed degeneration of the mucosa, hemorrhage into the mucous layer, accumulations of round cells and leucocytes just beneath the mucosa, and numerous accumulations of round cells scattered throughout the edematous submucosa.

From our experiments it becomes clear that the bacillus is not pathogenic when introduced into the blood stream. In our first communication we placed particular emphasis on the bladder lesions, since cystitis was observed in all "spontaneous" cases of the disease. Instillation of pure cultures of the bacilli obtained from the urine or kidney into the bladder of normal animals results in a well defined cystitis. It is true that the cystitis encountered under natural conditions is more severe, but the character of the urine and the gross and microscopic pathology are the same.

Ritzenthaler[5] called attention to the fact that the means by which cows become infected is unknown. We attempted to throw some light on this phase of the problem. Since we recognized that the

[5] Ritzenthaler, M., *J. Comp. Path. and Therap.*, 1910, xxiii. 33.

process is an ascending one, it seemed possible that the organism might inhabit the vagina of the normal cow or that it might be associated with metritis. The vaginal secretions of a considerable number of normal cows were examined bacteriologically. Diphtheroids were found, but in no instance could it be said that an organism identical with the pyelonephritis and cystitis bacillus was cultivated. The experimental inoculations of culture into the vagina confirmed these observations, for it was shown that the organism soon died when implanted on the vaginal mucosa.

It is conceivable that the bull during copulation might readily transmit the organism. The urine from 11 bulls was collected in rubber bags and examined. Diphtheroids were encountered in several samples but all differed culturally and immunologically from the specific bacillus. It seemed possible that the penis of the bull might become contaminated with the bacillus and thus the organisms gain access to the vagina of other cows. From the herd records we were able to obtain the dates on which known cases of the disease were bred, together with the record of the males' subsequent breedings. In this way we found three bulls that had bred 26 cows shortly after copulation with an infected cow. Urine examinations of these cows showed that one was infected. This cow occupied for several months an adjoining stall to a severe clinical case of the disease. In an isolated observation one bull served a cow suffering from a well marked cystitis. Within the next half hour the bull served three other cows. One of the cows was disposed of shortly after the breeding, but the other two remained uninfected. It is clear then that the bull is not a ready agent of transmission.

It is customary in the herd where most of our observations have been made to curry and brush the cows. Vigorous currying and brushing of the regions about the anus and vulva are done twice daily. This suggested itself as a means of transmission, especially since the bacilli might be implanted in tiny wounds or in exuded urine adhering to the vulva. An opportunity was afforded for observations on this point. Well defined cases of the disease were placed among four uninfected cows in such positions that the infected cows were brushed first. Two cows were disposed of within 1 month of exposure. From the urine of the third the bacilli were

isolated. This cow recovered. The fourth cow was found infected 5 months after the exposure was started.

DISCUSSION.

We concur with the opinion expressed by Joest[6] that pyelonephritis of cows is a specific infectious disease. The organisms that we have encountered in cases occurring on five different farms have been identical in morphology and cultural characters. This organism when introduced in small quantities into the bladders of normal cows promptly sets up cystitis which becomes chronic. The experimental disease is on the whole milder than that usually encountered in the herd, but the bacilli will persist in the urine for a period of months. In the case of the cows of our experiments it must be borne in mind that the animals were not pregnant and were fed only a moderate grain ration. Many have noted that certain forms of nephritis in cows may occur shortly after parturition. We have previously pointed out that statistics in the matter are liable to considerable error because of the possibility that there may have existed a long standing but unnoticed infection. However, we are inclined to believe that pregnancy may play a considerable part in increasing the severity of the disease. During the dry period the concentrates in the ration are diminished and a considerable bulk of dry matter is substituted. The amount of urine excreted is considerably less. The organisms will grow readily in normal cow's urine and abundantly in urine containing a little blood serum. Under the conditions of pregnancy, then, there is an ample opportunity for the bacilli to multiply and exert a relatively prolonged action on the bladder mucosa. Late in pregnancy the pressure on the bladder, and perhaps injury to the bladder during parturition, may serve to influence the disease unfavorably.

In certain respects the causative organism is unique. It possesses no pathogenicity for the smaller animals. As far as we can learn it inhabits only the urinary tract of cows, unlike other organisms associated with renal infection, such as *Bacillus coli* and *Bacillus pyogenes*. The organism cannot be said to be an inhabitant of the

[6] Joest, E., Spezielle pathologische Anatomie der Haustiere, Berlin, 1924, iii.

normal vagina or uterus and, as far as can be learned, plays no part in metritis. In our experiments in which large quantities of broth cultures were fed to cows, the bacilli never appeared in the feces. The lymph glands of the head and neck of a cow fed the organism were cultured with negative results. On the whole it can be said that it possesses but little pathogenicity for cows unless it finds its way into the urine where it readily grows and attacks the structures lining the bladder. The large numbers of bacilli in the urine in even mild cases strengthen the view that this is the method whereby the injury comes about.

Our experiments have thrown little light on the mode of dissemination of the organism. However, we feel that certain portions of the work are suggestive. In the first place, the bacilli attack only the mucosa of the urinary tract. They are not concerned in metritis. The disease is apparently independent of genital conditions. Once the bacilli gain access to the bladder they multiply and produce the characteristic cystitis. We feel certain that they do not reach the bladder by way of the blood stream, since cows injected intravenously with culture failed to develop cystitis. The experiments in which small amounts of culture were introduced into the vagina were negative. None of the cows became infected. Nevertheless, under most favorable conditions, organisms deposited in the vagina in the region of the urethra might very well find a favorable medium for growth and thence reach the bladder. When intimate contact occurs over long periods it has been possible to observe transient and prolonged infection, as in the experiment in which cows were placed in stalls adjoining clinical cases of the disease. Under these conditions the cows were curried and brushed vigorously twice a day and doubtless as a result the bacilli were mechanically deposited in the vagina at frequent intervals. From our observations we feel that the bull does not often act to disseminate the disease during copulation. Males are relatively insusceptible to the disease.

SUMMARY.

A Gram-positive diphtheroid isolated from spontaneous cases of cystitis and pyelonephritis of cows on five farms has been studied.

All the strains obtained were of the same general morphology. They possess similar cultural characteristics and to a great degree similar agglutination affinities. The organism is not pathogenic for the ordinary laboratory animals. It produces no toxin. Cows injected intravenously with broth cultures failed to develop the disease. On the other hand, small quantities of cultures introduced into the bladder by way of the urethra gave rise to a typical persistent cystitis.

There is evidence for a transmission of the disease by contact infection, as when the same individual curries both normal and infected cows.

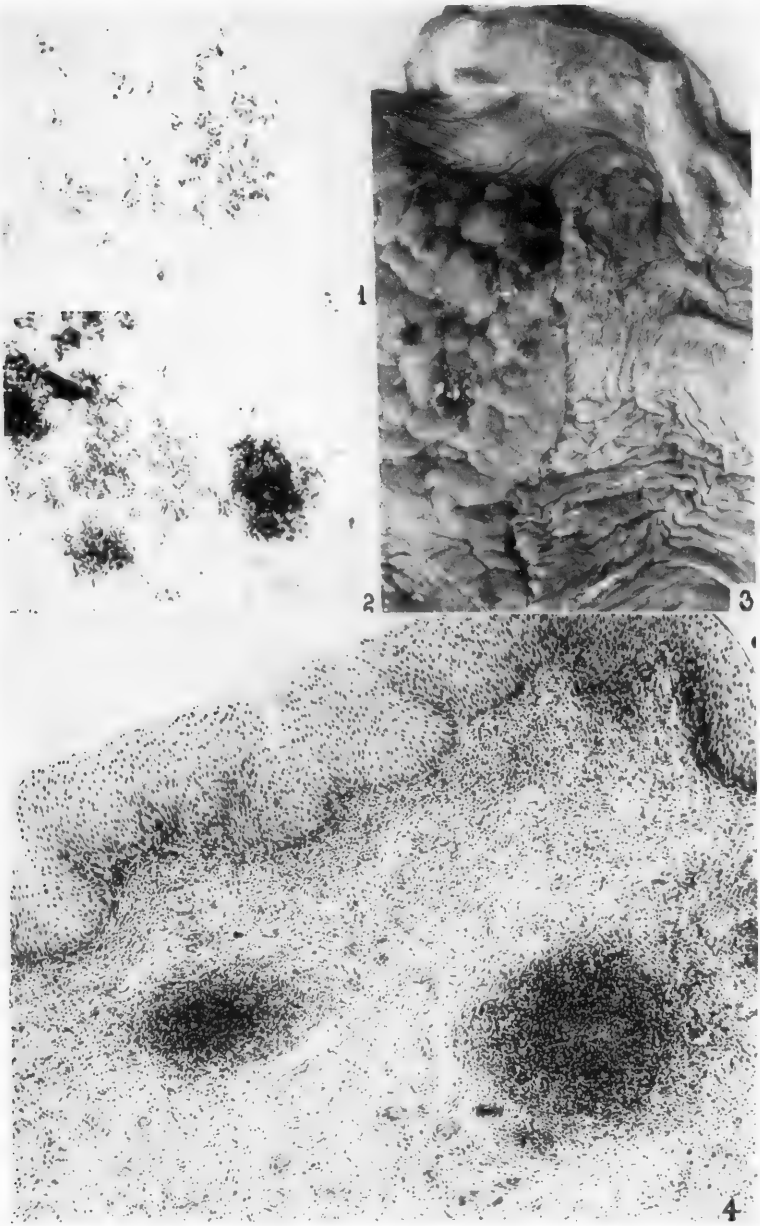
EXPLANATION OF PLATE 1.

FIG. 1. A 48 hour broth culture of the diphtheroid showing one of the characteristic aggregates. Methylene blue. × 880.

FIG. 2. The bacilli in the urinary sediment of Cow 1100, 17 days after inoculation of 3 cc. of broth culture into the bladder. Methylene blue. × 880.

FIG. 3. A portion of the bladder of Cow 1100, 43 days after artificial infection with culture. Note the swollen mucosa studded with dark areas of hemorrhage. About ⅔ actual size.

FIG. 4. A section of the bladder of Cow 1166 inoculated into the urethra with broth culture 100 days before autopsy. There is a marked infiltration of round cells in the lower portion of the mucosa and a well defined accumulation of round cells in the submucosa. The submucous connective tissue is hyperplastic and a proliferation of blood vessels has occurred. × 73.



(Jones and Little: Cystitis and pyelonephritis of cows.)

UNIV. OF MICH.

IMMUNOLOGICAL STUDIES WITH HERPES VIRUS WITH A CONSIDERATION OF THE HERPES-ENCEPHALITIS PROBLEM.

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I.

Since 1923 one of us has been attempting to transfer virus from human encephalitis cases to animals, using rabbits for most of the experiments and for a few of them monkeys. In the course of this work it has become necessary to make a thorough study of the virus of herpes febrilis, for the obvious reason that this virus has been identified with that of encephalitis lethargica by a great many experienced workers in the past (1). We will deal with our attempts to obtain a virus transmission from man to animals in a later section of this paper. Before approaching this phase of the problem, however, we wish to report briefly upon studies made with the herpes virus itself, and more particularly upon phenomena of immunity which can be experimentally studied with this virus more easily than with most other filterable agents. Moreover, some of the phenomena encountered in this study have, we believe, distinct bearing upon the encephalitis-herpes question.

Our work has been done with three strains of herpes, all of them isolated from vesicles on laboratory workers, some of them carried through several years. Unlike some of the British virus, our own strains have been extremely resistant when preserved in glycerol, one of them recently causing typical death in a rabbit, without material prolongation of the incubation time, after $11\frac{1}{2}$ months in 50 per cent neutral glycerol in the ice box.

Dosage of virus has been a matter to which we have given considerable thought, and in which we have tried to give attention not only

to the actual amounts of brain substance and dilution used, but also to the possibility that the virus might be irregularly distributed throughout the brain. In addition to the method of emulsifying weighed amounts of brain in definite volumes of salt solution, we have followed a method first used in this laboratory by Breinl, in which an entire brain or entire hemisphere was emulsified by grinding in sterile sand, gradual additions of salt solution being made up to 100 cc., and subsequent dilutions made from this. The dilutions in such procedure were calculated from the total brain as a basis. Thus one one-hundred-thousandth of such a dilution would indicate approximately a hundred-thousandth part of the entire brain.

In working with the virus, the interesting observation was made that if prolonged grinding in sand was carried out, and the suspension thus obtained was centrifuged at high speed for an hour or longer until the supernatant fluid was almost clear, rabbits inoculated with such supernatant fluid died often a day or two earlier than those inoculated with the sediment, indicating—to our minds—that in the brain the virus is intracellularly localized and that it is to some extent discharged from the cells in the process of trituration. It is not impossible that the intracellular position of the virus may have some bearing on the irregular results obtained by various observers in experiments upon *in vitro* neutralization by immune sera. Another way of obtaining virus relatively free from cells, which, however, we have not used in these experiments except to determine the possibility, is by tapping the cisterna magna when the animal begins to show symptoms, such spinal fluid being virulent at the time.

Active Immunization.

On this subject little can be added to what is already known, and our results have been consistent in themselves and in harmony with previous reports. The method we have used chiefly has consisted of a preliminary inoculation upon the cornea, followed by intracerebral[1] test doses at varying intervals. Active immunity has regularly followed survival from infection of the eye, appearing not earlier than 2½ weeks after the eye inoculation and lasting up to as long as 6

[1] For intracerebral inoculation ether anesthesia was used.

months. Later tests have not so far been made. Active immunity can also be obtained by preliminary skin inoculation as practised by Perdrau, and by intratesticular inoculation.

Neutralization of Herpes Virus with the Sera of Actively Immune Animals.

The literature on this question has been contradictory, some workers denying the neutralizing value of convalescent and immune sera; others, notably Flexner, having no apparent difficulty in such neutralization. Our first three experiments were entirely negative, although we titrated our infecting doses down to five times the minimum fatal amount. In some of these experiments, indeed, the rabbits inoculated with virus-serum mixtures died sooner than the saline controls. This we have now determined is due to a rapid deterioration of the virus when incubated with salt solution, a deteriorating effect prevented in the presence of normal serum.

These negative results agreed in general with those published by Perdrau, but were inconsistent with those of Flexner and others. We therefore continued with them, thinking that probably such differences might be due to fortuitous variations in the sera of individual actively immunized rabbits, depending perhaps upon the method of active immunization, the severity of the herpetic process, or the time factor.

Subsequent experiments were done by a simple technique in which about 50 minimum fatal doses of the virus, that is 0.1 cc. of a 1:10,000 dilution of fresh or glycerolated herpetic brain, were incubated with 1 cc. of either the active or inactivated serum of rabbits that had been immunized by a corneal inoculation and subsequently tested for immunity by an intracerebral one. The mixtures were kept at 37½° from 5 to 6 hours, and amounts of 0.25 cc., representing, thus, something over 10 fatal doses, were intracerebrally injected into normal rabbits. Controls with incubated saline and normal serum suspensions were used.

First Experiment.—The serum of the actively immunized rabbit was obtained 25 days after the second injection. In this experiment the saline control died in 9 days and the rabbit receiving the immune serum-virus mixture gradually lapsed into a lethargic condition from which it recovered in 3 weeks. This

rabbit will be discussed below. No normal serum control was made in this experiment.

Second Experiment.—The serum here used was taken 52 days after the second or intracerebral inoculation of the actively immune animal. The normal serum control died typically on the 4th day, the saline control after 6 days, and the immune serum animal died on the 8th day.

Third Experiment.—The serum used in this case was taken 59 days after the second or intracerebral virus inoculation of the immunized animal. The experiment was somewhat varied from the others in that both activated and inactivated immune and normal serum mixtures were made in order to determine any differences produced by inactivation. The rabbits receiving the mixtures in which immune serum, active and inactive, had been used survived; those in which normal active and inactive serum were used died on the 8th day, while the animal receiving the incubated mixture of virus and saline died after a protracted illness lasting 15 days, and the inactivated virus controls died on the 8th and 11th days, respectively.

From these and similar experiments we may conclude that the serum of rabbits actively immunized by corneal inoculation and subsequently reinjected intracerebrally develop definite protective bodies in their serum which probably do not appear sooner than the 3rd and 4th weeks in sufficient quantity to yield a clean-cut experiment.

Experiments like those above, however, are merely examples of the general type obtained. The results are irregular, thus accounting for discrepancies in the literature, but we do not believe that this need necessarily be accounted for by fluctuations in the potency of the sera themselves.

Irregularities are rather due, we believe, to the fact that in the ordinary technique of using brain material for virus, even when accurately measured dilutions are used, some of the virus is intracellular and some extracellular, and the serum protective bodies, whatever they may be, may therefore be blocked from complete contact with the intracellularly localized fraction. We have discussed this in our introduction, where we mentioned the fact that when virulent brain material is triturated for an hour in sterile sand, taken up in Ringer's solution, and centrifuged for an hour or more, the supernatant fluid, which is clear and relatively cell-free, kills a little more quickly than do equivalent volumes of the sediment.

To some extent the potency of sera, we think, may be enhanced by

what may be spoken of as hyperimmunization. We have treated a number of rabbits after they had developed active immunity with repeated intracerebral injections and large intraperitoneal injections of potent virus, and in this way have obtained sera which exhibited considerably more regularity in the neutralization of herpes virus. Comparative quantitative results cannot yet be made with sufficient accuracy to permit more than a very definite probability in favour of the enhancement of potency by such a method.

Immunization with Brain Extracts.

There has been some discussion in the literature concerning the possibility of the location of the immune substance in the suceptible cells; namely, those of the central nervous system. Here, again, there have been considerable contradictions, Perdrau (2), indeed, finding that the brain extracts of an immunized animal, far from immunizing, increased the virulence of the virus that had been in contact with it. His results in the latter respect may have been due to the salt solution effects that we have mentioned above.

Having determined that the brain suspensions of an immune animal do not retain active virus, we carried out a number of experiments analogous to those carried out with serum in which we incubated active virus with brain extracts of immune animals.

The brain extracts were made by grinding an entire hemisphere with sand for ½ hour, gradually adding 20 cc. of saline. The mixture was shaken for 2 hours, then set in the incubator for an hour, and again shaken for 15 minutes while warm. In some cases it was extracted in the ice chest for longer periods, but in others this was omitted. Finally, the material was centrifugalized at high speed and the supernatant fluid used.

First Experiment.—In this experiment the brain of an immune rabbit was taken out 74 days after the second injection and the rabbit bled at the same time. In other respects the experiment was carried out in a manner similar to that used with the serum tests. In this experment, curiously enough, there was no protection whatever by the brain extract, and the serum did nothing but prolong the life of the rabbit for 3 days beyond the control, and 2 days beyond the brain extract one, dying on the 8th day.

Second Experiment.—In this experiment, in which the brain was removed from the immune animal 62 days after the second inoculation, the result was approximately the same. Again, the serum protected, prolonging life until the 10th day, 4 days beyond the normal serum control; the brain extract rabbit dying on the 7th day.

These two experiments may therefore be regarded as practically negative.

The following experiment was carried out in connection with those on the protective properties of hyperimmunized rabbit serum.

The brain of a rabbit which had received an eye inoculation on August 31 and subsequently four intracerebral inoculations, the last one on December 21, was taken out 21 days after the last injection. Blood was obtained at the same time. The method of extraction was essentially similar to the one described above. Mixtures were made in which 1 cc. of the fresh brain extract was added, respectively, to 0.15, 0.1, and 0.05 cc. of a 5 per cent fresh virus suspension. A control was made of 1 cc. of the serum of the same rabbit with 0.2 cc. of the virus, and salt solution controls set up with 0.05 cc. of the virus, that is the smallest dose, mixed with the brain. After an incubation of 4 hours, 0.2 cc., respectively, of the mixtures was injected into rabbits. The control died in 9 days; the two rabbits receiving brain extract plus the larger doses of virus died in 5 and 10 days, respectively, while the rabbit receiving the smallest amount of virus plus brain extract, an amount equivalent to that received by the saline control, survived, as did the rabbit receiving the hyperimmune serum plus four times the amount of virus which killed the control and four times the amount of virus which killed one of the brain extract rabbits.

This experiment and another like it seem to prove conclusively that, while the brain extracts of hyperimmunized rabbits may contain a limited amount of protective substance, this is considerably less than that contained in the circulating blood of the same animal.[2]

Passive Immunization with Serum by Injecting the Serum Both Intravenously and into the Cisterna Magna the Day before Injecting the Virus.

All the experiments done in this direction up to the present time have been completely negative. Neither the intravenous administration of sera known to possess protective action in considerable quantities nor the injection of $\frac{3}{4}$ to 1 cc. of such sera into the cisterna magna the day before intracerebral infection has afforded even the slightest protection to the rabbits so treated.

[2] Incidentally a considerable number of complement fixations have been done in which filtrates of normal herpetic brains were used as antigen, and attempts have been made at agglutination reactions in virulent filtrates with the serum of immune animals. All of these efforts have remained entirely negative in result.

Are Rabbits Which Survive Neutralized Serum-Virus Mixtures Actively Immune?

In a number of experiments we have subjected rabbits that have survived serum-virus mixtures as above described to subsequent intracerebral inoculation with virus alone. In no case so far have these rabbits shown signs of resistance above the normal.

We report this type of experiment because we believe it adds additional proof to the other observations made that immunity does not follow unless there is an active process at least initiated in the animal during immunization.

Immunization with Phenolized Virus.

In considering the literature on immunity in connection with the filterable viruses, one gains throughout an impression consistent with the observations noted in this paper, that dead virus does not protect, or—in other words—that virus which does not give rise to at least a slight or moderate disease process is not followed by immunity. This, of course, is consistent with the many observations recorded in poliomyelitis, and observers who have studied this are unanimous in stating that monkeys who have failed for one reason or another to "take" may be actively infected in a subsequent experiment. The exception to this rule seems to be the now widely employed method of prophylactic rabies treatment by the Semple method, in which phenolized virus is subcutaneously administered. We have attempted to apply this method to protection against herpes virtually by the same method used by Semple in hydrophobia.

10 per cent suspensions, that is 5 gm. per 50 cc., of fresh herpes virus were made in a 1 per cent carbolic solution. This was incubated for 6 hours, shaken twice while incubating, and left in the refrigerator overnight. This was diluted on the following day with an equal volume of salt solution, making a ½ per cent carbolic suspension.

In attempting protection with phenolized virus made in this way, it seemed to us of the greatest importance to determine whether the carbolic acid killed or merely attenuated the active agent. This determination is incidentally of fundamental importance in appraising the immunological significance of the Semple method in rabies. We

therefore inoculated rabbits intracerebrally with 0.3 cc., respectively, of phenolized virus made as above, 6 hours, 36 hours, 48 hours, and 5 days after the phenol had been added. None of these rabbits, not even the one inoculated with the 6 hour phenolized material, showed any symptoms, even a temperature, and all remained healthy and well. None of them were immunized by the procedure. The only one that died was the 5 day one which died of diarrhea and pneumonia some time after the inoculation.

We can only conclude that the phenol used in the manner indicated by Semple kills the herpes virus.

Two series of three rabbits each were then treated with daily injections of the phenolized virus for 14 days. Test injections with virulent material were made 1, 2, and 3 weeks after the last dose of the phenolized material. In no case was there the slightest protection. Here again, therefore, we are in a position to conclude that no protection whatever is afforded by dead virus.

Believing that possibly the manner of administration might make a difference, we reinoculated intracerebrally some of the rabbits in which the phenolized virus had been administered in the same manner to test whether it was dead or not, and found that these rabbits, likewise, were unimmunized, even though the phenolized material had been given intracerebrally.

II.

The Herpes-Encephalitis Question.—In the course of the last 3 years we have attempted to transfer a virus from human encephalitis to animals with material of some eight cases.[3] In at least four of these the pathological examination seemed as reasonably certain in indicating encephalitis lethargica as this is possible, at the present time, by histological examination. In two of the cases where the possibility of poliomyelitis existed, this was ruled out by negative intracerebral monkey inoculation.

Experiments with Rabbits.—A large number of rabbits were used in these experiments, but we do not describe these inoculations in detail

[3] We have used spinal fluid from a number of other cases which, however, on later study could not be considered as representing probable encephalitic infection.

because, rigidly analyzed, they must all be regarded as negative. A good many of our rabbits died, and in some of the earlier cases we obtained a few rabbits that died in series up to the fourth generation from the original case. Careful autopsies and cultures in some of these cases revealed other possible causes of death and never have we obtained a typical herpetic syndrome in such animals. While a not inconsiderable number of the rabbits in these experiments died without morphological or cultural evidence of bacterial infection, our inability to carry on a virus in series, the complete absence of anything like an herpetic syndrome, and our later suspicions of possible spontaneous encephalitis justified us in rejecting any suggestions that we had killed any of these animals with a virus emanating from the human encephalitis case.

In our recent cases, in which the rabbits were more closely observed than before, we often obtained a type of languid and almost somnolent condition in many of the inoculated rabbits which was characterized by completely relaxed attitudes in the cages and immobility continuing for hours when the animals were not disturbed. Such animals, however, were always quite alert and normal when roused into activity either mechanically or by the proffer of food, and few of them died, even though observed for long periods. The two or three that did die usually had diarrhea, and transfer of material from their nervous systems did not produce disease in other animals. We believe it likely that the somnolent condition described may represent the clinical picture given by Kling and others which, however, as observed by us, was entirely unconvincing. We may summarize our attempts of directly transferring human encephalitic virus to rabbits with the statement that never, in spite of the use of a large number of animals, have we in any single instance produced anything simulating the herpetic syndrome in such animals, and the fortunate freedom of our experiments from spontaneous rabbit encephalitis has resulted in very few deaths of rabbits which in any manner would have tended to mislead us into thinking that we had made a successful transfer.

Attempt to Repeat Perdrau's Method of Repeated Inoculations.—Perdrau has recently described a procedure with which he reports successes in starting an herpetiform virus in rabbits by repeated inoculations of glycerolated encephalitic brain given on alternate days,

three inoculations to each rabbit, alternately on the skin, into the brain, and again upon the skin. He has varied this in a number of different ways. He also reports success with intracerebral inoculations of an encephalitis virus which had been preserved in glycerol for 43 days in cases in which the fresh, unglycerolated virus gave negative results. He furthermore succeeded by inoculating, together with the human encephalitic brain material, suspensions of the brain of herpes immune rabbits in which, by his own control tests as well as in repetitions of our own, no surviving herpes virus can be detected.

We tried Perdrau's method with material from three cases in two of which there seemed to be no possible question as to clinical or pathological diagnosis, and in one of these cases we persisted with the repeated inoculation technique quite beyond Perdrau's original report, administering in some cases as many as five injections. In no case did we either succeed in producing an herpetiform syndrome nor did we obtain any other type of disease in the rabbits which might have been attributed to a transmissible virus. Again, however, we noticed the prolonged somnolence, temporary loss of appetite, and that slight departure from normal which has been described above. In no case, however, did these rabbits develop the temperature changes which we have found so uniform in successful herpes inoculations.

In view of the failure of direct repetition of Perdrau's experiments, and considering the results with partial immunization of herpetic rabbits which will be recorded below, we thought it entirely logical to subject the animals treated by repeated injection of the encephalitic virus to subsequent herpes inoculation, with the idea of determining whether or not the development of a partial immunity would indicate the presence of perhaps an attenuated herpes-like virus in the encephalitis brains.

Altogether fourteen rabbits treated by the Perdrau method or some modification of it, all of them having received at least four consecutive inoculations of encephalitic material, were reinoculated with measured doses of herpes virus at periods sufficiently long after the last inoculation to warrant the expectation of some immunity. All these animals died at periods sufficiently close to those at which the controls succumbed to exclude the possibility of their having been even partially immunized by the injection of the encephalitic brain.

Partial Immunization.—Among the most interesting of the results of our experiments are those in which, either purposefully or by accident, the partial immunization of the rabbits to herpes virus was produced. These observations were begun a year ago in experiments carried out in this laboratory by Dr. Breinl.[4] They have since been extended in various ways. They are similar in result to some of those reported by Perdrau. Partial immunization was obtained either by the injection of sublethal doses of herpes virus, by which we mean the injection of 0.1 cc. of a 1:50,000 to a 1:200,000 dilution of a brain emulsion fatal in amounts of 1:10,000, or they were obtained by immunization with serum-virus mixtures. In one case the effects to be described were observed after the first inoculation, when a virus was injected which had been incubated with salt solution and thus attenuated, as described in a preceding section. It is not always possible to produce such rabbits at will, but when successful, the animals, after a slight preliminary temperature, develop gradual drowsiness and weakness at about the time that the controls die, and may go to sleep, remaining in this comatose condition anywhere from 4 to 8 days before death. Animals similar to these have been described by Perdrau. The brains of such rabbits are virulent.

One rabbit in this series is particularly interesting. This was a rabbit of about 2500 gm. which was injected on October 21 with 0.1 cc. of a 1:10,000 herpes virus obtained from the fourth brain passage of this particular material after 20 days in glycerol. The virus had been incubated for 5 hours at 37°C. before injection, which—as other experiments show—materially attenuates the virus. The animal at first showed no symptoms, but 3 weeks after inoculation it was found in a lethargic state, sleeping most of the time. Slight stimulation rapidly removed the drowsiness into which the animal at once relapsed as soon as stimulation was interrupted. The animal remained in this condition for 3 weeks, when a gradual recovery to normal occurred. During this entire course there was no fever, and careful examination elicited neither tremors nor paralysis. Muscular weakness seemed to be associated with the drowsiness, even when temporarily roused. On January 25, 1926, the animal was tested for immunity with 0.15 cc. of a 5 per cent virus suspension of a potent virus. 5 days later it developed a flaccid paralysis of the hind legs. There was no fever, but there was typical

[4]It was unfortunate that Dr. Breinl had to leave at a time when this work was started. Experiments referred to in the text were subsequently extended from observations made while he was still in this laboratory.

grinding of the teeth. A control done with the same virus at the same time died on the 30th with typical symptoms. On January 31 the condition took the form of a gradually ascending paralysis, the animal lying on its side and still moving its front legs, the head slightly bent to one side, as with a spasm. This condition continued throughout February 1. Death occurred on February 2, the entire clinical picture from beginning to end being as parallel as possible in clinical story with Case 9 of our human encephalitis series.

These experiments were considered of great importance because, in spite of all of the preceding negative evidence, the clinical picture in these cases is so strikingly similar to the conditions observed in diseased human beings that they rather open the possibility that the encephalitis and herpes virus may still be related, but that in passing through the nervous system of man and remaining there for some time, the herpes virus may have become so attenuated for rabbits that it can no longer be started in these animals unless exceptionally fortunate cases are encountered. Such exceptional circumstances might explain the ease with which earlier investigators obtained rabbit transmission, as contrasted with the negative results of Flexner and Amoss' (3) experiments and our own. Moreover, it is not at all impossible that the positive findings of herpes virus in the central nervous systems of human beings not suffering from encephalitis, as well as the innocuousness of encephalitis virus administered to men, as in Busacca and Bastai's (4) experiments, might be explained by an immunity to the virus existing in many people. For it is more than probable that human beings may develop a considerable resistance against the deeper penetration of a virus so ubiquitous in our environment and so frequently causing lesions of minor importance on the skin and mucous membranes. We mention these things because it is necessary in a subject as important as this one to consider all points of view as broadly as possible. Moreover, the extraordinary attenuation of fixed rabies virus for man furnishes a closely pertinent analogy.

SUMMARY.

In the preceding experiments observations have been reported upon the nature of herpes virus which confirm the suspicion that the virus is intracellularly located in the infected nervous system.

In regard to the immunological conditions existing in this disease, our experiments have reaffirmed that herpes virus can be neutralized with the serum of actively immunized animals and have offered an explanation for the irregularity of the results of others, as well as our own.

It has been found that brain extracts possess some virus-neutralizing power, but considerably less than the serum of the corresponding animals.

Attempts at passive immunization with neutralizing serum were uniformly negative, even when the serum was introduced into the cisterna magna 12 to 24 hours before infection with the virus.

It has been shown that active immunity can be attained only when some degree of reaction to the living virus has occurred. Rabbits which survived neutralized serum-virus mixtures did not acquire immunity nor did those treated with virus phenolized to the extent of actual destruction. This point suggests a reinvestigation of the Semple method of rabies immunization.

In so far as our studies touched upon the herpes-encephalitis problem we have uniformly failed in attempts to transfer herpes virus directly from man to rabbits. These results are in contradiction to those of most of the earlier workers, but in keeping with the recent reports of Flexner and Amoss.

Attempts to overcome the difficulty of transfer by the recently published technique of Perdrau were unsuccessful. Furthermore, animals repeatedly treated with human encephalitis material, either fresh or glycerolated, as practised in the Perdrau method, failed to acquire the slightest degree of immunity to subsequent herpes inoculation.

By the inoculation of very small doses or by infection of partially immunized rabbits, as described above, we have succeeded in modifying the characteristic herpetic syndrome in rabbits in a manner which simulates many of the clinical features of human encephalitis.

Our own experience forces the conclusion that no valid proof exists upon which can be based an assertion concerning the identity of the virus of herpes with that of encephalitis lethargica. Either the two viruses are entirely unrelated, or else prolonged sojourn in the central nervous system of man attenuates the virus for rabbits to an extent

analogous to that in which rabies virus is attenuated for man by passage through rabbits. The isolated successes of Levaditi and of Doerr and their assistants might thus be regarded as fortunate exceptions in which material incompletely attenuated had been at their disposal.

We suggest this point of view as an alternative working hypothesis largely because the results we are reporting, as well as those of Flexner and Amoss, are in flat contradiction to the reported successes of earlier workers and the more recent experiments of Perdrau. The experiments of the latter, as described, cannot be explained by the occasional existence of spontaneous encephalitis in his rabbits, nor by the assumption that a herpes virus fortuitously coexisted with that of lethargic encephalitis in his material, inasmuch as this material alone at first injection or in the unglycerolated state failed to infect. It is also possible to conceive that human beings may, by repeated skin infections, attain a not inconsiderable partial immunity to herpes virus, which would explain the nature of the clinical course (as in our partial immunity rabbits) as well as the innocuousness of direct injections of herpetic virus into man, as reported by Bastai and Busacca, and the finding of herpes virus in human beings not suffering from lethargic encephalitis.

These suggestions are discussed in order to give this important problem the broadest possible consideration.

For the time being, however, such reasoning cannot be taken as more than a logical possibility impressed upon us by our partial immunization experiments.

All other experimental evidence obtained by direct inoculations with the limited material at our disposal tends to render identity of the two varieties of viruses unlikely.

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THE SKIN RESPONSE OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

I. Description of a Secondary Reaction Occurring Locally after Intradermal Inoculation.

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Plate 2.

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INTRODUCTION.

The present paper contains a description of a remarkable and unexpected series of events which followed the inoculation of non-hemolytic streptococci into the skins of rabbits. Following the studies of Rivers (1) on the skin lesions produced in rabbits by the intradermal inoculation of hemolytic streptococci, we applied similar methods with the non-hemolytic streptococci, but immediately encountered a phenomenon quite different from anything described by previous observers with any type of bacteria. In brief, it was found that the intracutaneous inoculation of rabbits with certain non-hemolytic streptococci produced a raised lesion, often 20 to 30 mm. across, which reached its maximum size in 24 to 48 hours and then began to retrogress; about the 8th or 9th day, however, an increase in the size and redness of the lesion occurred in many animals. This period of increased activity lasted for 2 or 3 days; then the lesion gradually disappeared. This phenomenon has been designated the "secondary reaction;" it seemed to us worthy of detailed study.

EXPERIMENTAL.

Technique.—Adult rabbits weighing from 1200 to 2500 gm. were used. The hair on their flanks was clipped short with scissors and then completely removed with a solution of sodium sulfide consisting of 35 gm. to 60 cc. of water. As a rule this depilation was carried out several days before the animals were inocu-

lated. Parallel experiments showed that the cutaneous responses to inoculation were similar whether the skin was prepared by this method of depilation or by shaving with a razor. 18 hour cultures of bacteria in 0.5 per cent rabbit blood broth were used for inoculation; plain broth cultures gave comparable results; but since the streptococcus most studied, Strain V92, grew very poorly in plain broth, it was thought best to use rabbit blood broth throughout. In most experiments the cultures were made in 50 cc. of broth; the growth was centrifuged and the sedimented organisms made up to 1 cc. with normal salt solution. Thus 0.1 cc., the quantity usually inoculated, contained the organisms from 5 cc. of the original culture. Inoculations were made intradermally on the dorsal portion of the rabbits' flanks. The two diameters of the lesions were measured with calipers daily by the same observer. The size of any lesion is represented in the charts by the sum of these two diameters (see Chart 1).

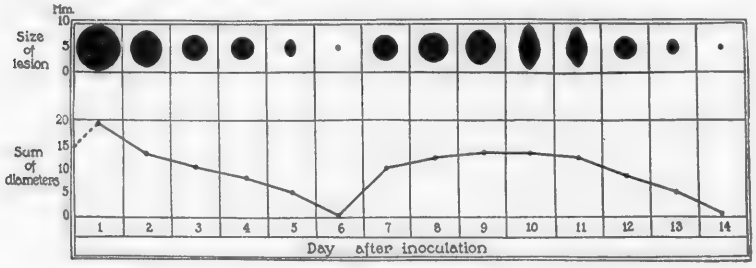

CHART 1. Showing results of a small inoculation of green streptococcus (V92) in a rabbit's skin. Above, the actual size of the lesion from day to day is represented. Below, is plotted a curve of the variations in size, when the sum of the two diameters of the lesion is taken as the index.

Early in the work most of the inoculations were with one strain of *Streptococcus viridans* (V92) which had been isolated from a subcutaneous fibroid nodule of a patient with rheumatic fever.

Primary Reaction.—When the deposit from 5 cc. of a culture of this strain was inoculated as described, a lesion of fairly uniform character resulted; after 24 hours its diameters were in the neighborhood of 30 by 20 mm.; it was raised 3 or 4 mm. above the surface of the surrounding skin; it was red, especially towards the middle; and near the dorsal end there was usually a small yellow or greenish center. Surrounding this central nodule was a definite zone of edema and erythema which fused gradually with the surrounding skin. As a rule the size of the

lesion ceased to progress after 36 to 48 hours. A rapid diminution in size then began; the surrounding edema decreased rapidly and the central nodule more gradually. This nodule often became covered by a scab after 2 or 3 days, usually without much purulent discharge. Not infrequently, however, there was neither scab formation nor breaking down of the skin over the lesion. Where no secondary reaction occurred the nodule continued to retrogress; commonly it was no longer palpable after 3 weeks.

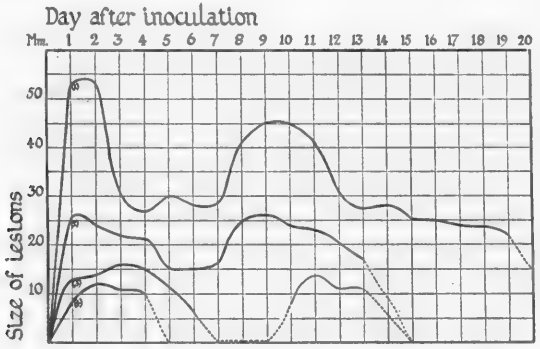

CHART 2. Showing types of curves with secondary reactions when a rabbit was inoculated intradermally at the same time in four different places with different sized doses of Streptococcus V92, as follows:

Curve (1) sediment from 5 cc. of blood broth culture.
" (2) 0.1 cc. of blood broth culture.
" (3) 0.01 " " " " "
" (4) 0.001 " " " " "

Secondary Reaction.—In more than half of the rabbits, however, a change set in about the 8th or 9th day (see Fig. 1). The whole lesion, which had become fairly pale, took on once more a bright pink color and began to enlarge with an average increase of 3 or 4 mm. in each diameter; but, at times, the increase took place in only one direction, usually ventralward, but sometimes dorsalward, or laterally. Frequently small outlying red satellite nodules appeared; these sometimes fused later with the main lesion. Occasionally a lesion at the time of the secondary reaction attained a size greater than it had immediately following

inoculation. Doses of 0.1, 0.01, and 0.001 cc. of streptococci produced proportionately smaller primary lesions. With 0.1 cc. doses the lesion never disappeared completely before the onset of the secondary reaction, and the secondary reaction usually occurred in the same animal simultaneously with that seen in lesions produced by larger doses. With 0.01 cc. doses, however, the primary lesions usually disappeared after the 4th to 5th day, but in the rabbits having a secondary reaction there was a recurrence of an erythematous papule slightly later than the time of the maximum secondary reactions seen in larger lesions. No secondary reaction has been observed at the site of inoculation of 0.001 cc. (see Chart 2). No lesion ever broke down at the time of the secondary reaction. The secondary reaction lasted from 1 to 3 days and then subsided. The total duration of a lesion following 5 cc. or 0.1 cc. inoculations was from 1 to 2 weeks longer in those that showed a secondary reaction compared with those that did not. A definite third period of activity was not observed.

The secondary reaction did not occur in all rabbits inoculated with Streptococcus V92 (see Fig. 2). Of 151 injected with living cultures of this organism, 79 showed a definite secondary reaction and 54 failed to show it; in 18 there was a very slight increase of only 1.5 or 2 mm. in each diameter; hence, it was doubtful whether or not a true secondary reaction occurred; reactions of this order are classed as "doubtful" in the tables. Increases in the size of a lesion of 1 mm. or less in each diameter are recorded as negative. According to whether the "doubtful" group is, or is not, counted as positive, either 52 or 64 per cent of the rabbits may be said to have shown secondary reactions following inoculations of Streptococcus V92. The occurrence or non-occurrence of a secondary reaction appeared to be independent of the age, sex, and color of the rabbits. Since secondary reactions did not occur in 100 per cent of normal rabbits it was found necessary to use three or more animals for every experiment and for every control in order, as far as possible, to avoid false conclusions.

While the height of the secondary reaction was usually on the 8th or 9th day following inoculation, it also occurred not uncommonly on the 7th or 10th day, rarely earlier or later than this. The actual figures for the day of maximal secondary reaction in 117 animals are as follows:

4th day.....	2 rabbits.	10th day.....	23 rabbits.
5th "	2 "	11th "	9 "
6th "	5 "	12th "	6 "
7th "	11 "	13th "	4 "
8th "	23 "	14th "	1 "
9th "	30 "	16th "	1 "

= 117 rabbits.

The lesions produced were studied both bacteriologically and histologically. On several occasions we inoculated rabbits intradermally in six or eight places and subsequently excised a lesion every day under ether anesthesia. These lesions were studied by staining direct films, by making cultures, and by histological methods.

Bacteriological Studies.—The films, stained by Gram's method, showed many polymorphonuclear leucocytes from the beginning and, after the 6th day, some macrophages as well. Streptococci were seen in large numbers at first but became progressively fewer, until they were usually no longer visible after the 6th or 8th day; on two occasions, however, they were still seen on the 10th and 12th days respectively. They were often found phagocyted either by polymorphonuclear leucocytes or by macrophages; it must be supposed that they were usually dead, as they were not infrequently seen in films when they could no longer be recovered in cultures. In making cultures the excised tissues were pinned out on cork with the epidermal surface downwards, and a portion of the lesion was removed under aseptic conditions from the exposed surface without cutting through the epidermis. We thus hoped to avoid contamination, but, even with this technique, a few colonies of contaminants, especially staphylococci, appeared from time to time in the cultures. The excised tissue was finely divided with scissors, ground with sterile sand and normal salt solution with a pestle and mortar, and inoculated both into broth and into poured blood agar or dextrose blood agar plates. Streptococci always grew plentifully from 1 day old lesions, less abundantly from those 2 days old, and occasionally none were obtained from lesions 3 days old. On three occasions, however, a growth was obtained 7 days after the rabbit was inoculated, and once after 9 days. There was never any evidence of an increase in the number of streptococci at the time of the secondary reaction; in fact, streptococci were never recovered from lesions at the time of a secondary reaction.

Histological Study.—The tissues were fixed in Zenker solution containing acetic acid and stained with eosin and methylene blue and with Gram's stain. The histological changes were as follows:

24 hours after inoculation the lesion consisted of a mass of polymorphonuclear leucocytes and bacteria forming a small abscess with ill defined walls. Edema and hyperemia were present in the surrounding tissues. A number of mononuclear cells, apparently formed as a result of vascular endothelial proliferation, were commonly seen around the small blood vessels in the deepest part of the lesion. During the next 3 days, granulation tissue gradually formed outside the mass of polymorphonuclears, until by the 5th or 6th day the central abscess was fairly well shut off by a wall of young connective tissue. This wall was more marked in lesions that followed the larger doses, and less in the smaller lesions. Rather regularly about the 6th day numbers of macrophages appeared at the periphery of the mass of disintegrating polymorphonuclears and apparently acted as scavengers. In lesions removed from rabbits which showed no secondary reactions in duplicate areas not excised, there appeared on the 5th and 6th days collections of small round cells—both lymphocytes and plasma cells—about many of the blood vessels a short distance from the abscess wall. Similar collections of cells were also present in the young connective tissue making up the abscess wall. These collections of small round cells became more distinct for 2 or 3 days, then gradually decreased as the fibrous tissue making up the wall became more dense. In the tissues of animals which showed a secondary reaction similar collections of small round cells were not observed until the end of the secondary reaction.

At the time of the secondary reaction a distinct type of histological response was noted.

In the small blood vessels, especially in the arterioles just outside of the granulation tissue there was a marked swelling of the endothelial cells which assumed a cuboidal shape and gave the vessel the appearance of an epithelial lined gland duct. Spaces around some of the vessels were also packed with large polygonal endothelioid cells having a basophilic protoplasm; in other places masses of similar cells were seen not as intimately associated with blood vessels. Numerous isolated swollen connective tissue cells were also present in the subcutaneous tissue, corium, and between the muscle fibers more distant from the abscess wall. In these more remote zones the connective tissue fibers were more widely separated than normal, probably the result of edema.

In satellite nodules excised at the height of the secondary reaction there was a central area made up of a mixture of pycnotic and fragmented nuclei, polymorphonuclear and mononuclear cells, and amorphous granules. This was surrounded by masses of polygonal endothelioid cells, and by blood vessels showing marked endothelial proliferation. A moderate number of polymorphonuclears were present in this zone. In secondary reaction tissue a few days older the

endothelioid reaction became less marked, masses of small round cells appeared in the perivascular regions, and the tissues gradually assumed the appearance seen in the simple lesion which did not show secondary reaction.

Bacteria Giving Rise to a Secondary Reaction.—It was naturally of interest to know just what bacteria could produce the secondary reaction described. Was this property peculiar to the strain of streptococci used, was it common to all green streptococci, or would it follow inoculation of other microorganisms? Ten other strains of *Streptococcus viridans*, all isolated from subcutaneous nodules, blood, or hearts of patients with rheumatic fever, were accordingly studied. Five of these gave rise to well marked secondary reactions in 50 per cent or more of animals tested. No secondary reaction, or at most a doubtful one, followed inoculation of the other five strains; it is true that they were not tested on large series of rabbits. Details are given in Table I. At first we failed to induce this reaction with *Streptococcus viridans* isolated from sources other than rheumatic fever; two strains from normal throats gave no definite secondary reaction; three strains from the blood of patients with *Streptococcus viridans* endocarditis gave negative results; a fourth strain (T4), however, from a patient with subacute bacterial endocarditis induced two definite and two doubtful secondary reactions. In this patient the endocarditis was grafted upon a congenital heart lesion, and there was no evidence of past or present rheumatic fever.

A few other streptococci were studied. One (T106), which was indifferent to blood and came from the urine of a patient with nephritis, caused secondary reactions in all three rabbits tested, even though there were very trifling primary reactions. An anomalous streptococcus (T27B), which was isolated from a subcutaneous rheumatic fibroid nodule caused hemolysis on rabbit blood agar plates but not in liquid media containing blood and fermented mannitol but not lactose; this organism produced a secondary reaction in two out of four rabbits.

Because of the close relationship between *Streptococcus viridans* and the pneumococcus it was of special interest to test the capacity of the latter to induce secondary reactions. Intracutaneous inoculations of rabbits with living virulent pneumococci produced large necrotic lesions and caused the animals to die in 3 to 5 days. It was, therefore, necessary to use heat-killed virulent pneumococci or aviru-

TABLE I.

The Incidence of Secondary Reactions in Rabbits Injected Intradermally with Various Bacteria and Bacterial Products.

Type of bacteria.	Strain.	Source.	No. of rabbits.			
			Inoculated.	With definite secondary reaction.	With doubtful secondary reaction.	Without secondary reaction.
Living:						
Green streptococcus	V92	Rheumatic fever.	151	79	18	54
" "	V110A	" "	8	4	0	4
" "	V110B	" "	4	4	0	0
" "	V119	" "	2	2	0	0
" "	38D	" "	8	3	1	4
" "	A49	" "	2	1	0	1
" "	A135	" "	2	0	1	1
" "	W72	" "	3	0	0	3
" "	T27h	" "	5	0	1	4
" "	T20a	" "	2	0	1	1
" "	T20b	" "	2	0	0	2
" "	A148	Bacterial endocarditis.	2	0	0	2
" "	W67	" "	2	0	0	2
" "	T57	" "	3	0	0	3
" "	T4	" "	9	2	2	5
" "	T51	Normal throat.	4	0	0	4
" "	T52	" "	4	0	1	3
Indifferent streptococcus	T106	Nephritis.	3	3	0	0
Avirulent pneumococcus	Type IR	—	13	2	0	11
Hemolytic streptococcus:						
Anomalous "	T27b	Rheumatic fever.	4	2	0	2
Bovine "	Manhattan.	—	3	0	0	3

Human streptococcus	S3	Bronchopneumonia.	6	0	0	6
" "	NY5	Scarlet fever.	1	0	0	1
" "	T43	Rheumatic "	3	0	0	3
" "	S23	Lobar pneumonia.	3	0	0	3
" "	T10	Rheumatic fever.	3	0	0	3
" "	T40	" "	3	0	0	3
Staphylococcus	Various.	—	21	1*	1	19
Micrococcus catarrhalis	—	Normal throat.	4	0	0	4
B. coli	—	" stool.	6	0	0	6
Heat-killed:						
Green streptococcus	V92	—	17	6	0	11
Virulent pneumococcus	Type IS	—	9	1	1	7
Avirulent "	" IR	—	6	0	0	6
Hemolytic streptococcus	S3, S23	—	9	0	0	9
Products of green streptococci:						
Nucleoprotein	T4, V92	—	9	0	0	9
Soluble substance	T4	—	4	0	0	4
Culture filtrate	V92	—	10	0	0	10

* See text (page 44).

lent living forms.[1] In thirteen animals tested with avirulent living cultures (R form) there were two secondary reactions, on the 11th and 14th days respectively; six tested with similar cultures heat-killed (56°C. for 1½ hours) showed no secondary reactions. Virulent Type I (S form) heat-killed in a similar manner induced one definite and one doubtful secondary reaction in a total of nine rabbits tested; these reactions occurred early—on the 5th day. It seems, therefore, that the pneumococcus has the capacity of inducing secondary reactions, but this is less marked than in some strains of green streptococci.

Bacteria Giving Rise to No Secondary Reaction.—In striking contrast to the other members of the streptococcus group, inoculation of typical hemolytic streptococci uniformly failed to be followed by a secondary reaction. Six strains, a scarlet fever streptococcus included, were tested on nineteen rabbits. It may be mentioned that two out of three of these died within 2 days of receiving the scarlet fever streptococcus—a result which never followed the injection of any other streptococcal strain. It must be borne in mind that the inflammation resulting from hemolytic streptococcus injection was considerably more extensive than that caused by green streptococcus; this fact in itself may have had some influence on the subsequent course of events. One strain of bovine hemolytic streptococci was tested on three rabbits without causing any secondary reaction.

With one exception, injections of staphylococci never led to the production of a secondary reaction. Four strains were tested on twenty-one rabbits. The exception is of some interest in that it occurred in a rabbit which was inoculated at the same time with green streptococcus (V92); when the streptococcus lesions increased in size the staphylococcus lesions increased simultaneously. The experiment was repeated without reproducing this result; hence its significance is not clear. *Micrococcus catarrhalis* failed to induce a secondary reaction in any of four rabbits; *Bacillus coli* did not do so in any of six rabbits tested.

Results of Second Inoculations.—It naturally was of interest to determine whether or not this secondary reaction would appear re-

[1] For the cultures of avirulent pneumococci we are indebted to Dr. H. A. Reimann who produced these "rough" forms from virulent "smooth" forms by various manipulations (see Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587).

peatedly in one animal. A rabbit, having received an intradermal inoculation of 5 cc. green streptococci and a subsequent similar inoculation of the same strain within a period of 9 weeks, showed primary reactions of approximately the same intensity following both injections, but within this period never gave a secondary reaction after the second injection. This was equally true whether the second inoculation was on the same or the opposite side of the animal. Whenever previously inoculated rabbits were retested with Strain V92, equal numbers of normal animals were used as controls in order to be certain

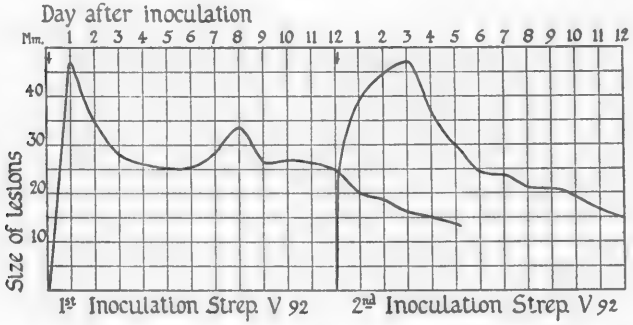
Day after inoculation
Mm.
1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12
40
30
20
10
Size of lesions
1st Inoculation Strep V 92
2nd Inoculation Strep V 92

CHART 3. Showing that a rabbit which gave a secondary reaction after first inoculation with Strain V92 failed to give a secondary reaction after the second inoculation with the same strain. Arrows indicate times of inoculation.

that the strain still regularly induced a secondary reaction. Thus twenty rabbits which had received an intradermal inoculation of Strain V92 from 1 to 9 weeks previously all failed to show a secondary reaction following a later injection (see Chart 3). The same result was obtained with two rabbits inoculated with Strain V110A and two with Strain V110B; all four had shown well marked secondary reactions after their first inoculations. This inhibiting effect was not specific; a previous injection with any green streptococcus, whether or not it itself produced a secondary reaction, sufficed to prevent a reaction occurring after a later injection with Strain V92 (see Chart 4). Thus Table II shows that this inhibiting effect occurred after preliminary treatment with Strains T51, T52, and T27h. Six intravenously im-

munized rabbits also failed regularly to show any secondary reaction. The duration of this altered reactivity of the animal—we hesitate to call it immunity—was not thoroughly studied. Rabbits do, however, regain their ability to show secondary reactions after a period of at least 9 weeks, for among thirteen animals inoculated more than 9 weeks after their first injection six showed no secondary reaction, two a doubtful, and five a definite reaction.

Typical hemolytic streptococci, it will be recalled, never gave rise to secondary reactions; nevertheless a previous injection with living cul-

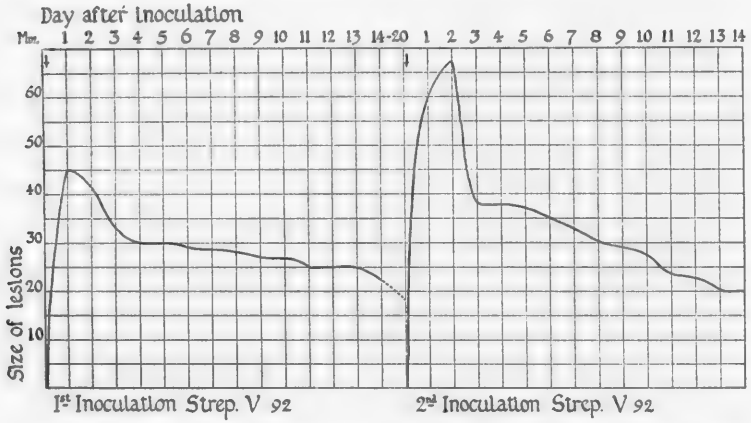
Day after Inoculation
Mm. 1 2 3 4 5 6 7 8 9 10 11 12 13 14-20 1 2 3 4 5 6 7 8 9 10 11 12 13 14
60
50
40
30
20
10
Size of lesions
1st Inoculation Strep. V 92
2nd Inoculation Strep. V 92

CHART 4. Showing that a rabbit which did not give a secondary reaction after first inoculation with Strain V92 also failed to give a secondary reaction after the second inoculation with the same strain. The inoculation with any streptococcus has a similar protecting effect.

tures of these organisms regularly inhibited the development of a secondary reaction when a green streptococcus, such as Strain V92, was inoculated into an animal a few weeks later. This inhibition was seen four times after intradermal inoculations of hemolytic streptococci and six times after the rabbits had been inoculated with hemolytic streptococci into subcutaneous agar foci. In nine animals which received a preliminary injection of heat-killed hemolytic streptococci there was one definite and one doubtful reaction following inoculation with Strain V92.

Preliminary inoculation with pneumococci, on the other hand, was less effectual in preventing secondary reactions after a later injection with green streptococci. Thus among a total of twenty-eight rabbits first inoculated with pneumococci, six gave definite and two doubtful reactions after later inoculations with Streptococcus V92. Similar results were obtained whether the pneumococci were living or heat-killed Type IR or heat-killed Type IS.

Inoculations with microorganisms not belonging to the streptococcus group, on the other hand, appeared to be unable to prevent the second-

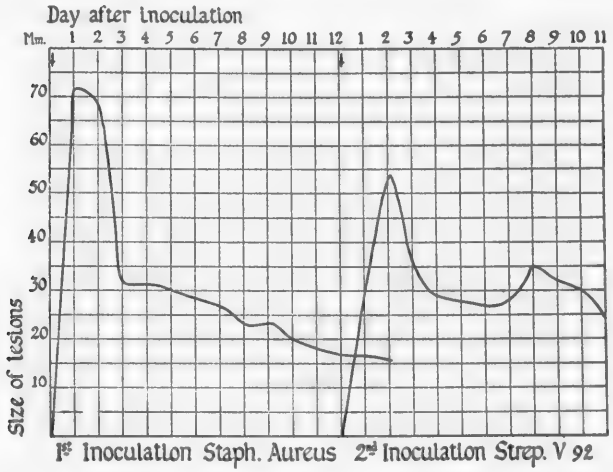
Day after Inoculation
Mm. 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11
70
60
50
40
30
20
10
Size of lesions
1st Inoculation Staph. Aureus 2nd Inoculation Strep. V 92

CHART 5. Showing that a preliminary inoculation with *Staphylococcus aureus* does not prevent a secondary reaction after inoculation with Streptococcus V92.

ary reaction (see Chart 5). Of nine rabbits injected first with staphylococci and within 9 weeks with Streptococcus V92, four showed good secondary reactions; similar results are recorded in Table II in animals previously treated with *Bacillus coli* and with *Micrococcus catarrhalis*.

Results of Inoculations with Killed Bacteria.—The results of cultures from the lesions indicated that the secondary reaction was not due to an increased activity on the part of the microorganisms injected. Cultures of green streptococci (V92) were killed by heating at 56°C. for an hour or by 1 per cent phenol and then shown to be sterile; when

TABLE II.

Effect of Various Bacteria and Bacterial Products in Preventing the Appearance of a Secondary Reaction When Green Streptococci Were Subsequently Injected.

First inoculation.			Subsequent inoculation.			
				No. of rabbits.		
Bacteria.	Strain.	Production of secondary reaction.	Strain*	With definite secondary reaction.	With doubtful secondary reaction.	Without secondary reaction.
Living:						
Green streptococcus.....	V92	+	V92	0	0	20
" "	Intravenous V92	−	V92	0	0	6
" "	V110A	+	V110A	0	0	2
" "	V110B	+	V110B	0	0	2
" "	T27h	0	V92	0	1	2
" "	T4	+	V92	0	0	3
" "	T51	0	V92	0	0	2
" "	T52	0	V92	0	0	2
" "	V92	+	†V92	4	2	5
" "	V110B	+	†V110B	1	0	1
Pneumococcus avirulent.....	Type IR	+	V92	1	1	11
Hemolytic streptococcus.....	S3	0	V92	0	0	3
" "	NY5	0	V92	0	0	1
" "	T10 agar foci.	−	V92	0	0	6
Staphylococcus.....	—	0	V92	4	0	5
Micrococcus catarrhalis.....	—	0	V92	1	1	1
B. coli.....	—	0	V92	3	0	3
Heat-killed:						
Green streptococcus.....	V92	+	V92	2	0	10
Virulent pneumococcus.....	Type IS	+	V92	3	1	5
Avirulent "	" IR	0	V92	2	0	4
Hemolytic streptococcus.....	S3	0	V92	1	1	4
" "	S23	0	V92	0	0	3
Products of green streptococci:						
Nucleoprotein.....	T4, V92	0	V92	2	0	3
Culture filtrate.....	V92	0	V92	2	1	8

*All subsequent inoculations were within 9 weeks of the first inoculation unless indicated with the sign†.

inoculated as usual into rabbits, they gave rise to lesions similar to those caused by live cultures and not very much smaller. Of seventeen rabbits so inoculated, six showed secondary reactions as marked as were usually seen following live cultures (see Table I). These killed cultures were demonstrated also to be almost as effective as live ones in preventing secondary reactions from following a later dose of live organisms. This prevention was complete in ten out of twelve animals; and in the other two a small secondary reaction occurred. Nine rabbits were injected with hemolytic streptococci killed at 56°C. without showing a secondary reaction; but upon reinjection with Streptococcus V92 one showed a definite and one a doubtful reaction.

Since the whole group of streptococci was apparently concerned with the phenomenon under study and since Lancefield (2) had shown that the nucleoproteins obtainable from various members of the group are antigenically related, we studied lesions produced by injection of some nucleoproteins derived from two strains, T4 and V92, which she kindly gave us. These products produced definite lesions in the rabbits' skins, but induced no secondary reaction in any of nine rabbits; nor did nucleoprotein injections prevent the appearance of a secondary reaction in response to a subsequent inoculation of live streptococci after an interval of 12 or 15 days. No secondary reaction was ever seen to follow the injection into rabbits' skins of soluble precipitable substance (residue antigen) prepared by Dr. Lancefield from a green streptococcus (T4), nor did it follow the injection of broth filtrates of streptococcus cultures. The subcutaneous or intradermal injection of these filtrates, moreover, did not prevent the occurrence of secondary reactions when the rabbits were inoculated intradermally with living cultures of Streptococcus V92 after 5 to 17 days.

Results in Guinea Pigs.—Twelve guinea pigs were inoculated into the skin of the shaved abdomen with strains of green streptococci (V92, V110A, V110B) known to produce secondary reactions in rabbits: in no guinea pig was a definite secondary reaction observed within a period of 2 weeks; they were not observed longer. Tests have not as yet been made on other laboratory animals.

DISCUSSION.

A phenomenon referred to as a "secondary reaction" in rabbits' skins has been described; it can be regularly produced in a certain percentage of rabbits; its interpretation offers difficulties which have not as yet been surmounted. It is felt, however, that some of the possibilities which immediately suggest themselves can be definitely excluded. Our natural thought at first was that the bacteria originally injected showed an increased activity at the time of the secondary reaction, or that the defensive mechanism of the host had temporarily broken down. The first idea at least was negatived by our failure to recover streptococci from the lesions at the time of the secondary reaction and by the appearance of the reaction after the introduction of killed bacteria. It does not seem probable, moreover, that the phenomenon is due to secondary infection, because frequently the lesions did not ulcerate at any time, and they commonly failed to yield any growth on culture. The staphylococci occasionally encountered were never numerous. If these possibilities are excluded, there are three possible sources for a hypothetical substance which might excite the secondary reaction. This substance may be: (1) a product of the streptococci; (2) a product of the animal host; (3) a product of the interaction of the streptococci and animal host.

The failure of the reaction to appear until the 8th or 9th day makes one hesitate to accept the first of these three possibilities. Films show that streptococci are progressively decreasing in numbers in the lesion from the 2nd day onward. It is difficult to explain why a toxic product contained in the bacteria themselves should not exert its action earlier than the 8th day. It is, however, possible, that the disintegration of the streptococci or degradation of the bacterial substance to a stage where toxic material is liberated is a process requiring several days. A toxic product of the rabbits' tissues themselves cannot be excluded; if such exists it would seem to be a substance only called into existence at this particular time by a certain class of bacteria. The third possibility, that of a toxic product formed by the interaction of substances furnished by both invader and host, is more inviting. Analogies with serum sickness in man and the Arthus phenomenon in rabbits are at once obvious; and if we accept this interpretation, we

are almost compelled to classify the secondary reaction as an allergic phenomenon. We may suppose that the streptococci or some antigenic product of them persists at the site of inoculation until after the formation of antibodies by the rabbit, and that, just as in the Arthus phenomenon, the product of the antigen-antibody union, which then occurs, excites a local inflammation. A number of difficulties appear when one attempts to prove this hypothesis experimentally. They will be discussed in a later paper. Whatever may be the eventual explanation of the secondary reaction, certain features in it are of great interest. The failure of the phenomenon to appear after a second inoculation, at any rate if this is made within a limited period after a first, makes it very probable that the substance exciting the reaction is antigenic. During this refractory period the rabbit may possess an immunity to the irritating substance of such sort that it gives no secondary inflammatory response; or antibodies present in the tissues as a result of the first inoculation may unite immediately with the antigen, producing an inflammation which is masked by the more vigorous response of the animal to the injected streptococci; the primary and secondary reaction would thus occur simultaneously and be superimposed.

The most interesting feature of the phenomenon is its particular association with the streptococcus group. Since preliminary injection with any member of that group, in which we include the pneumococci, usually results in the prevention for a certain period of the occurrence of the secondary reaction following the inoculation of a strain ordinarily capable of exciting this reaction, it is probable that some preventing or antigenic substance is one common to the whole group. Whether this substance and that exciting the secondary reaction are one and the same is a matter for future study.

It may be that this secondary reaction is a curious phenomenon of academic interest only. On the other hand, there is much yet to be learned of the part which streptococci play in disease. The study of this skin response of rabbits may enable us to interpret the relationship between streptococci and their host from a new angle. Attempts have often been made to explain rheumatic fever, nephritis, and some of the sequelæ of scarlet fever in terms of allergy, particularly in relation to streptococci. This reaction, especially if it should prove to be a

form of allergy, may eventually have some bearing on complex problems such as these.

SUMMARY.

1. Rabbits inoculated intradermally with certain strains of green streptococci have well marked lesions which, after reaching a maximum size in 24 to 48 hours and then beginning to retrogress, show in over 50 per cent of the animals a secondary increase in size and other signs of inflammation about 8 or 9 days after inoculation.

2. This secondary reaction may follow the inoculation of a variety of strains of green-producing streptococci from various sources, of indifferent streptococci, and occasionally of pneumococci.

3. The inoculation of hemolytic streptococci, staphylococci, *Micrococcus catarrhalis*, or *Bacillus coli* has not been followed by this secondary reaction.

4. The secondary reaction is not due to increased activity of the injected bacteria since the lesions are usually sterile at the time it occurs and since the secondary reaction occurs after inoculation of killed organisms as well as after that of living ones.

5. A second inoculation of green streptococci into a rabbit within 9 weeks of a first injection is followed by a primary but not by a secondary reaction. This inhibition of the secondary reaction is not specific; for the phenomenon of secondary reaction can be completely inhibited by previous inoculations of the rabbit with any living streptococcus and usually with pneumococci. Killed organisms are less effectual.

6. Preliminary injection with staphylococci, *Micrococcus catarrhalis*, and *Bacillus coli* has not prevented the appearance of a secondary reaction in response to subsequent injections of green streptococci.

7. The agent responsible for the secondary reaction has not as yet been determined; its nature is discussed. The phenomenon is possibly a form of allergic reaction.

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EXPLANATION OF PLATE 2.

FIGS. 1 and 2. Lesions in two different animals 8 days after inoculation.

Each animal was injected at Site I with 0.1 cc. and at Site II with 0.02 cc. of culture on the same day.

The comparable lesions in the two animals were similar until the 7th day, but on the 8th day Animal 1 (Fig. 1) showed a marked secondary reaction in each lesion, whilst in Animal 2 (Fig. 2) there was no secondary reaction at any time.

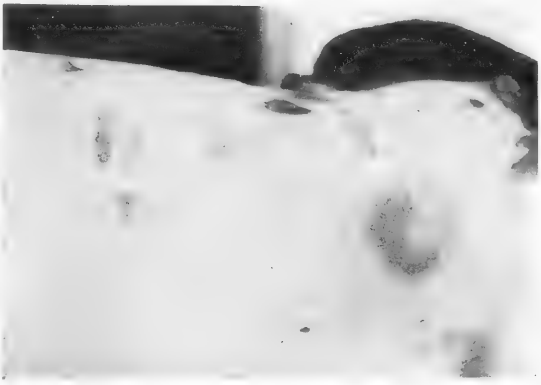

FIG. 1.

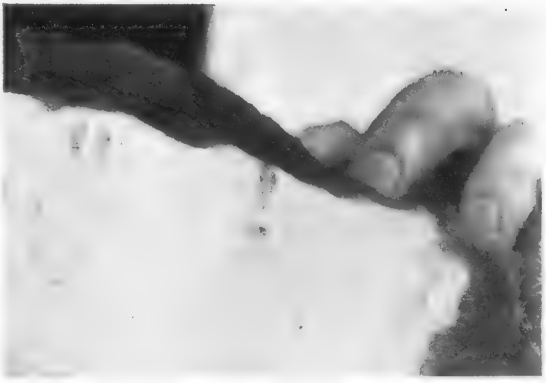

FIG. 2.

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THE SKIN RESPONSE OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

II. Attempts to Determine whether the Secondary Reaction Is of the Nature of an Arthus Phenomenon.

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INTRODUCTION.

In the preceding paper are described the lesions which follow the inoculation of certain non-hemolytic streptococci into the skin of the rabbit; such lesions begin to decrease in size after 24 to 48 hours but in over 50 per cent of animals show a recrudescence of activity about the 8th or 9th day. This phenomenon we have termed the secondary reaction. It usually fails to occur in animals which have been previously inoculated with any variety of streptococcus within a period of 9 weeks. Reasons have been given for attributing this secondary reaction either to a toxic product of the streptococci which for some reason takes a number of days to exert its action, or more probably to an antigen-antibody reaction, analogous perhaps to the Arthus phenomenon.

Arthus (1) noted that rabbits immunized against horse serum would show an acute local inflammatory reaction in response to a subcutaneous injection of horse serum which produced no such effect when injected into a normal animal. In a recent study of this phenomenon, Opie (2) concluded that the inflammatory reaction depended upon the formation of a toxic product when antigen and antibody were brought together in suitable proportions, because he found that the intensity of the Arthus phenomenon was roughly parallel to the intensity of precipitin formation. An inflammatory reaction resulted not only when antigen was injected into an animal the body fluids of which contained antibody, but also when antibody was injected locally into a rabbit which had very recently received an injection of antigen and which probably still contained that antigen in its body fluids; acute inflammation also followed the subcutaneous injection of an *in vitro* mixture of antigen and antibody.

Although it was shown by us that no bacteria could be grown from the skin lesions at the time of the secondary reaction, still it is easily comprehensible that antigen in the shape of dead microorganisms or degradation products thereof might still be present locally. With the knowledge that antibodies usually are first demonstrable about 8 days after a primary inoculation the hypothesis naturally suggested itself that the secondary reaction might be the result of the union of these antibodies and some locally persisting antigenic substance, which formed a new irritating compound. The fact that this reaction was demonstrable in rabbits gave additional support to an analogy with the Arthus phenomenon. In considering this hypothesis we fully realize that it is not always possible to apply our knowledge of other forms of allergy to that obtained with bacteria and bacterial products.

This possible analogy between the secondary reaction and the Arthus phenomenon seemed open to experimental study. If similar conditions held good in the two cases we should expect to find (1) that injections of streptococcal antigen into an animal having antibodies in its body fluids would provoke an inflammatory reaction not shown by a normal animal; (2) that injection of antibody into an animal the fluids or tissues of which contained streptococcal antigen would provoke an inflammatory reaction; (3) that mixtures of streptococci and antibody would give rise to more inflammation than was caused by either alone; (4) that some quantitative relation would be found between the occurrence of a secondary reaction in an animal and the antibody formation by that animal.

The technique used in the following experiments was the same as that described in the preceding paper.

EXPERIMENTAL.

I. Attempt to Alter the Reaction of a Rabbit's Skin to Green Streptococci by Giving Homologous Immune Serum Locally before Inoculation.

Two rabbits received intradermally ½ cc. of immune rabbit serum produced by weekly intradermal immunization; six injections in all had been given in the process of immunization and the resulting agglutinin titre of the serum against Strain V92 was 1:2560. On the following day live green streptococci, Strain V92, were inoculated into the site of the serum injection. The lesions resulting were of

approximately the same size (50 and 55 mm.) as in two control animals (52 and 53 mm.). Moreover, the serum had apparently no inhibiting effect on the development of a secondary reaction, as one of the two animals had a typical secondary reaction at the usual time, that is on the 9th day.

II. Attempt to Precipitate a Secondary Reaction by Giving Immune Serum Intravenously a Few Days before the Reaction Was Due.

Two rabbits were given an intravenous injection of 5 cc. of antistreptococcus immune serum similar to that used in Experiment I, 5 days after they had received an intradermal injection of green streptococci (V92). No immediate reaction occurred at the site of the skin lesions. One of the two showed a secondary reaction at the usual time (10th day); the other showed no secondary reaction. Since it was possible that 5 cc. might be an insufficient quantity of serum, two other rabbits were each given 45 cc. of immune serum intravenously 4 days after intradermal inoculation; this serum was also prepared by intradermal immunization of rabbits, but had an agglutinin titre of only 1:640 against Streptococcus V92. Results similar to those observed in the first group were obtained. Neither animal showed an accelerated secondary reaction; one showed no secondary reaction; the other gave a somewhat late reaction on the 13th day.

III. Attempt to Precipitate a Secondary Reaction by Giving Immune Serum Locally a Few Days before the Reaction Was Due.

By giving immune serum at the site of the lesions, it was hoped to obtain a greater local concentration of antibodies than was possible after intravenous passive immunization. Three animals were, therefore, injected intradermally in two places with green streptococci; 5 days later, one rabbit, and the following day the other two, were each given 0.2 cc. of homologous immune serum at the site of one lesion and 0.2 cc. of normal rabbit serum at the site of the other. Aside from the slight local reaction usually seen in any rabbit when serum is injected intradermally, no rabbit showed any immediate response suggesting a premature secondary reaction. Two of the rabbits showed no secondary reaction; the third showed typical reactions in both lesions on the 9th and 10th days respectively.

IV. Effect of Injection of Mixtures of Streptococci and Immune Serum into Normal Rabbits' Skins.

The sediment of 5 cc. blood broth culture of green streptococci, Strain V92, was mixed *in vitro* with immune sera prepared by intravenous or intradermal inoculation of rabbits with the homologous organism. The mixture was inoculated into four rabbits. For controls, mixtures of bacteria with saline, bacteria with normal rabbit serum, and bacteria with heterologous green streptococcus immune

serum were used. The maximum size of the lesions at the end of 24 hours is shown in Table I.

It is evident from Table I that the lesions resulting from mixtures of bacteria and immune serum were no larger than those following inoculation of organisms alone, or of mixtures of organisms and normal

TABLE I.

Effect on the Size of the Lesions Produced in Rabbits' Skins of Mixing Various Sera with the Streptococci Injected.

Rabbit No.	Serum used.	Size of lesion (sum of diameters).
		mm.
1	None.	39
	Normal rabbit.	38
	Homologous streptococcus.*	27
	" " †	32
2	None.	47
	Normal rabbit.	36
	Homologous streptococcus.*	30
	" " †	26
3	None.	39
	Normal rabbit.	35
	Homologous streptococcus.*	34
	Heterologous " *	29
4	None.	43
	Normal rabbit.	34
	Heterologous streptococcus.*	21
	" " †	21

* Immune serum produced by intravenous inoculation.
† " " " " intradermal "

serum. They were, in fact, always slightly smaller than the controls. Measurements of these lesions at the end of 48 and 72 hours, and of lesions resulting from inoculation of the sediment from 0.5 and 0.05 cc. of culture with the same amount of serum confirmed these results. If this is evidence of any protective power in the serum it was apparently not specific. This point was, however, not studied in detail.

V. Attempts to Correlate the Appearance of the Secondary Reaction with the Production of Immune Bodies.

We next endeavored to determine whether immune bodies could be demonstrated in a rabbit's serum after one intradermal injection of the sediment from 5 cc. blood broth culture streptococci, and whether the degree of antibody formation was related in any way to the second-

TABLE II.

Relationship between Agglutinin Production and Occurrence of Secondary Reaction.

Rabbit No.	Green streptococcus used.	Secondary reaction.	Day on which secondary reaction was maximal.	Agglutinins present.					
				Before inoculation.	Days after inoculation.				
					5	8	9	10	11
1	Strain T4	+	10	0	0	.	++	.	.
2	" V110A	+	11	.	.	.	++	.	.
3	" V110A	+	10	.	.	.	0	.	.
4	" V92	+	8	0	.	.	.	0	.
5	" V92	+	5	0	.	.	.	0	.
6	" V92	+	9	0	.	0	0	.	0
7	" V92	±	10	0	.	0	++	.	++
8	" V92	±	9	0	.	.	.	0	.
9	" V92	±	7	0	.	.	.	0	.
10	" V110A	±	10	.	.	.	0	.	.
11	" T4	0	—	0	+	.	++	.	.
12	" V110A	0	—	0	0	.	0	.	.
13	" T4	0	—	0	0	.	0	.	.
14	" V92	0	—	0	.	.	.	0	.
15	" V110A	0	—	0	0	.	0	.	.

In the agglutinin column ++ means agglutination up to 1:80 to 1:160.

ary reaction. Agglutinins were chiefly studied; for obtaining stable suspensions of streptococci we used the technique described by Dochez, Avery, and Lancefield (3).

The results summarized in Table II indicate that there was no absolute correlation between the ability of a rabbit to give a secondary reaction and its power to form agglutinins in demonstrable concentration. Thus, in only two out of six rabbits showing a definite secondary reaction was it possible to demonstrate agglutinins on the

TABLE III.

Relationship between Agglutinin Production and Occurrence of Secondary Reaction.

Rabbit No.	Time of bleeding with reference to inoculation. Days before.	Days after.								Relative strength of agglutinin production.
	1	5	6	7	8	9	10	11	14	15	
1	1	3	.	4	.	9	.	12	.	17	12
	.	+++	+++	++++	++++	+++	+++	+	.	.	
2	0	.	1	.	8	.	2	.	7	.	18
	.	+	++++	+++	+++	++	+	.	.	.	
3	4	.	12	.	13	.	19	.	14	.	2
	.	.	.	++++	++++	++++	++++	(e)	.	.	
4	0	.	9	.	4	.	9	.	11	.	15
	.	.	.	+	++	++++	++++	±	.	.	
5	0	.	.	10	.	13	.	11	.	17	8
	.	.	.	.	++	++++	++++	++++	.	.	
6	0	.	.	13	.	13	.	18	.	6	3
	.	.	.	+	+++	+	±	.	.	.	
7	0	.	16	.	15	.	11	.	11	.	5
	.	.	+	++	±	±	.	.	.	.	
8	2	.	.	14	.	19	.	19	.	15	1
	.	.	.	.	++	++	+	+	±	.	
9	4	.	.	9	.	15	.	18	.	11	4
	.	.	.	++	+	.	.	.	.	.	
10	0	.	3	.	7	.	10	.	9	.	14
	.	.	.	+	++	.	.	.	.	.	

Agglutinin strength indicated by figures (see text). (?) means serum lost.
Intensity of secondary reaction indicated as follows:

0 indicates no secondary reaction.
± " an increase of less than 3 mm.
\+ " " " " 4– 6 mm.
++ " " " " 7– 9 "
+++ " " " " 10–12 "
++++ " " " " 13 mm. or more.

(e) means lesion excised.

TABLE III—*Concluded.*

Rabbit No.	Time of bleeding with reference to inoculation. Days before.	Days after.									Relative strength of agglutinin production.
	1	5	6	7	8	9	10	11	14	15	
11	0	.	.	10	.	8	.	10	.	6	13
	.	.	.	.	.	+	++	.	.	.	
12	0	3	.	7	.	(?)	.	13	.	6	11
	.	.	.	.	++	.	.	.	.	.	
13	0	.	1	.	2	.	4	.	6	.	20
	.	.	.	.	+	+	+	+	.	.	
14	0	.	6	.	9	.	15	.	12	.	6
	.	.	.	.	+	+	.	.	.	.	
15	0	.	9	.	11	.	13	.	17	.	10
	.	.	.	.	.	+	+	.	.	.	
16	0	.	10	.	13	.	12	.	12	.	9
	.	.	.	.	.	+	±	.	.	.	
17	0	.	.	3	.	(?)	.	1	.	2	21
	.	.	.	.	.	±	+	.	.	.	
18	0	3	.	3	.	1	.	9	.	5	17
	.	.	.	±	±	.	.	.	.	.	
19	1	.	.	13	.	14	.	(?)	.	11	7
	.	.	.	.	±	.	.	.	.	.	
20	0	.	7	.	9	.	8	.	13	.	16
	.	.	0	0	0	0	0	0	0	.	
21	0	.	1	.	1	.	6	.	6	.	19
	.	.	.	0	0	0	0	0	.	.	

9th or 10th day; in one out of four rabbits giving a doubtful secondary reaction equally as strong agglutinins were present on the 9th and 11th days, while the other three developed no agglutinins at the time of the secondary reaction. Among five rabbits not showing secondary reac-

tions one gave a good agglutinin titre, the other four gave none. While a lack of absolute correlation between these two reactions is indicated by this table, there seems to be some rough parallelism between the proportion of rabbits giving both reactions positive and both negative. The criticism can, however, be made that the sera of these animals was not tested frequently enough to demonstrate immune body formation.

A more extensive single experiment was, therefore, performed to test this point.

Twenty-one rabbits were each inoculated in two places with the sediment of 5 cc. of 18 hour culture of Strain V92 and the course of the skin reaction charted as usual. All of the animals were bled before inoculation; half of them were subsequently bled on the 6th, 8th, 10th, and 14th days, the other half on the 7th, 9th, 11th, and 15th days respectively; when secondary reactions occurred before this period the rabbits were also bled at this earlier time. All of the sera were stored in the cold, and tested simultaneously for agglutinins against the same suspension of Strain V92. Each serum was diluted 1:20, 1:40, 1:80, 1:160, and incubated 2 hours at 56°. Immediate readings were made and recorded. Tests repeated on 2 different days confirmed one another. For purposes of comparison the number of + marks obtained with each serum were added, the sum multiplied by the factor 2 to eliminate fractions, and the final product entered as the intensity of agglutinin formation for each animal on a given day. While this method is not absolute it does give a good comparative index of agglutinin content of each serum. The intensity of the secondary reaction is charted by a series of pluses. We thus have a comparison of the agglutinin curve in each rabbit with the intensity of the secondary reaction over its entire period.

In Table III the results obtained with various animals are arranged from above downward according to the time of appearance, intensity, and duration of the secondary reaction in each rabbit. In the right hand column is shown the comparative intensity of agglutinin formation of each rabbit during the period of observation. Although a very rough correlation between the intensity of the two reactions may possibly be seen, a detailed analysis indicates that three of the five rabbits having the most marked secondary reactions were below the median in agglutinin formation; while four of the seven rabbits showing a + or ± secondary reaction were above the median. It is unusual for such a small proportion of rabbits to have no second-

ary reaction; hence the experiment may be criticized from the standpoint of negative control; but the results in general indicate a lack of correlation between the formation of humoral antibodies and the intensity of the secondary reaction.[1] This was also confirmed by testing these sera for precipitins against nucleoprotein and soluble specific substance prepared from Strain V92, when a complete disagreement between precipitin formation and secondary reaction was found.

DISCUSSION.

In five different types of experiments we have attempted to test the hypothesis that the secondary reaction might be explained on the lines of a local anaphylaxis comparable with the Arthus phenomenon. In none did we obtain any support of this hypothesis. No accelerated reaction was brought about in infected animals by introducing immune serum intravenously or locally; antigen-antibody mixtures produced no unusual reactions; no relation could be found between the secondary reaction and agglutinin or precipitin production.

In view of what has already been demonstrated by Zinsser (4) and his coworkers concerning the tuberculin reaction and its relation to antibody formation on the part of a tuberculin-sensitive animal, it was perhaps too much to expect that the Arthus phenomenon would find a close analogy in the cutaneous phenomena associated with an infection. In one case we are dealing with a coagulable protein, in the other with bacteria or bacterial products; hence we are not justified in concluding from our negative results that the secondary reaction is not allergic in nature. It is, indeed, well recognized that two types of hypersensitive reactions may be demonstrated in the skins of animals: in one the response is rapid and largely exudative in nature, best exemplified in the urticarial wheal; in the other the response is slower in appearance and characterized by more proliferation of the fixed tissue elements. We are unable to state from our experiments that the proliferative response seen at the time of the secondary reaction is not

[1] It is probable that the difference in the number of negatively reacting animals summarized in Tables II and III is due to the fact that the first group received a single inoculation while the second group received two. We have recently demonstrated that results are much more uniform when two inoculations of 5 cc. each are given.

due to the union of some product of the bacterial cell and an antibody present in the tissue cells, which antibody we are unable to recognize and work with in the same manner as we can with the usual humoral antibodies. In any event it would seem that the secondary reaction is analogous rather to the tuberculin reaction than to the Arthus phenomenon, and experiments are now under way with this last viewpoint in mind.

SUMMARY.

An attempt has been made to interpret on the lines of the Arthus phenomenon the secondary reaction which follows the intradermal inoculation of certain non-hemolytic streptococci into rabbits; but evidence in support of this interpretation of the reaction has not been obtained. The facts make it seem probable that if this secondary reaction is due to a hypersensitive state it must be one more closely allied to the tuberculin reaction than to the Arthus phenomenon.

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THE FILLING AND EMPTYING OF THE GALL BLADDER.

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PLATE 3.

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The discovery of a means of visualizing the gall bladder by the Roentgen ray after intravenous injection of certain halogen derivatives of phenolphthalein by Graham and Cole (1) has given a new means of studying the physiology of the biliary tract. This article belongs to a series of experimental and clinical reports (2) relating to that subject and will be particularly concerned with the filling and the emptying of the gall bladder. The experimental work has been done by both cholecystography and other laboratory methods.

A resistance to the outflow of bile from the common duct was found by cholecystography to be necessary for the collection of bile by the gall bladder (3). This resistance to the outflow of bile from the common duct into the duodenum was later found to be largely due to tonus and movements of the duodenum (4). As a result of this regulatory mechanism at the distal end of the common duct there are considerable changes of pressure in the gall bladder and the rest of the biliary tract which not only aid in the filling of the gall bladder but are a factor in its emptying. In this article we shall refer to this mechanism for the regulation of the flow of bile at the distal end of the common duct as the common duct sphincter.

The pressure changes that are reflected over the rest of the biliary tract by the opening and closing of the common duct sphincter have been demonstrated in the etherized dog in the following way.

A cannula which was inserted into the middle hepatic duct was connected with a supply of physiological saline solution for making pressure in the system. The continuous flow of bile in the hepatic ducts from the liver of man and dogs is under secretory pressure. A tambour connected by a T-tube with the cannula

was used to record changes of pressure in the hepatic duct on a smoked drum. Records of changes of pressure were also made by tambours connected with the gall bladder and with the common duct. Lateral hepatic ducts were ligated. When the common duct is closed, as if by the action of a sphincter, the pressure in the duct quickly rises. After a short delay, the pressure in the gall bladder rises. The pressure in the hepatic duct rises simultaneously with that in the gall bladder. If the common duct is opened, there is an immediate fall of pressure in it which is followed shortly and consecutively by a fall of pressure in the gall bladder and hepatic duct. We have recorded a drop of pressure in the gall bladder occurring at the same time with the relaxation phase of a peristaltic movement in the duodenum. This experiment was performed by ligating the hepatic ducts and simultaneously recording the pressure in the gall bladder and peristalsis in the duodenum.

These changes of pressure in the biliary tract are exactly similar to the changes of pressure that have been demonstrated by one of us (5) in a model designed to show the changes of pressure which normally take place in the biliary tract.

The control of the contents of the gall bladder by the common duct sphincter is further demonstrated by cholecystography.

The stomach or duodenum of man or animal must be free from food in order to secure a cholecystogram after administration of sodium tetraiodophenolphthalein. If digestion is in progress, bile is permitted to enter the duodenum too freely by the common duct sphincter; and the iodine in the bile will not enter the gall bladder to produce a shadow. Likewise, a cholecystogram is not obtained if the common duct sphincter of the dog is kept open by the insertion of a cannula into the distal end of the common duct. The bile laden with the halogen passes directly through the hepatic and common duct to the duodenum. A cholecystogram may be obtained, however, after the ligation of the hepatic duct of a middle lobe of the liver of a dog. The bile from the other lobes, containing the iodine, enters the common duct from the lateral hepatic ducts, which, in the dog, enter the common duct distal to the cystic duct, and is dammed back into the gall bladder by the closure of the common duct sphincter.

A factor in the prevention of the overdistension of the gall bladder by continuous accessions of bile is the S-shaped portion of the neck of the ampulla and cystic duct. Attention has been called to this fact by Jacobson and Gydesen (6). When there is considerable pressure in the fundus of the gall bladder, the S-shaped portion kinks upon itself and tends to prevent the exit or the entrance of more bile. The valves of Heister in the cystic duct do not normally seem to offer much resist-

ance to the entrance or exit of bile. They are doubtless more efficient as a control when the kink of the cystic duct is present. The ampulla and cystic duct are of great clinical importance.

4 or 5 hours after the intravenous injection of sodium tetraiodophenolphthalein, sufficient iodine will enter the normal gall bladder of a fasting man or dog to cast a shadow on a film by the Roentgen ray. The shadow in 4 or 5 hours becomes smaller and is densest between 16 and 24 hours after injection. The shadow tends to come to a minimum size and gradually fades away by the end of 30 to 40 hours.

We found when investigating the appearance and disappearance of the shadow of the gall bladder that the halogen in the bile is concentrated two to four times by the gall bladder (3). This fact confirmed the work of Rous and McMaster (7) who demonstrated the concentration of bile pigment by the absorptive power of the gall bladder. It was further found that the greater part of the contents of the gall bladder leaves its lumen by way of the cystic duct (3). This latter fact has also been demonstrated by Whitaker (8). However, this conclusion is in contradistinction to the opinions of Sweet (9), Halpert (10), and Demel and Brummelkamp (11), who believe that whatever enters the gall bladder through the cystic duct does not normally pass out of it by way of the cystic duct.

Factors of dilution seem of great importance in the appearance and disappearance of the shadow of the gall bladder. If all of the hepatic ducts are ligated, leaving the common duct intact, after a dense shadow of the gall bladder has been secured from tetraiodophenolphthalein, the shadow will be found to persist for many days even while food is being taken. It is inferred from this experiment that dilution of the bile in the gall bladder by bile entering from the hepatic ducts is an important factor in the filling and emptying of the gall bladder. Ordinarily, during a cholecystographic examination, the course of events is somewhat as follows:

The tetraiodophenolphthalein is excreted in the bile shortly after injection. The sphincter of the common duct diverts a considerable portion of the bile into the gall bladder. The amount of bile secreted is probably not large, comparatively, since the animal is fasting. The addition of iodine to the bile in the gall bladder proceeds. The gall bladder becomes distended. At this time we may obtain our first shadow of the gall bladder. It is a large and faintly visible one.

The amount of iodine in the gall bladder increases by new additions of bile as the concentrating activity of the gall bladder proceeds simultaneously. The iodine content becomes great enough to cast the densest shadow in 16 to 20 hours after injection of the dye. During this same period, the iodine has probably been entirely excreted by the liver. Since iodine is no longer present in the bile coming from the liver, the shadow gradually disappears as dilution of the iodine in the gall bladder progresses. In the meantime, food has been given to the patient and the disappearance of the shadow is hastened by an increased secretion of bile and a relaxed common duct sphincter. The backing up of dilute bile from the liver into the gall bladder mechanically aids in the expulsion of the concentrated bile from the gall bladder.

Changes of density of the cycle of the shadows of a gall bladder that normally occur after administration of tetraiodophenolphthalein may be duplicated outside of the body by the use of a rubber bag containing sodium iodide. A small thin rubber bag approximately the size of a gall bladder is attached to the arm of a T-tube. The changes in the shadows of a gall bladder can be duplicated by varying the size of the bag and by the dilution of its content of sodium iodide.

It was suggested by the preceding experiments of ligating the hepatic ducts and by other facts that the gall bladder is never entirely empty. Such was found to be true. The shadow of the gall bladder of a dog will always be present if he is given a daily dose of tetraiodophenolphthalein and films made at intervals. It persists even though he is fed regularly and allowed normal freedom of action. The shadows vary in size but tend to reach a minimum size.

The finding that the gall bladder is never empty is consistent with pressure changes in the biliary tract. The pressure in the gall bladder tends to come to an equilibrium with the rest of the system though it is not necessarily the same. Bile will pass out from a distended gall bladder if the common duct sphincter is open. Also, if the sphincter is open, bile coming from the hepatic ducts may assist in the emptying of the gall bladder both by dilution and by tending to produce a negative pressure in the gall bladder in its passage down the ducts through the open sphincter into the duodenum, in a manner similar to the action of the filter pump. When its content is at a minimum pressure the gall bladder will not discharge bile even though it is not entirely empty.

The fact that the gall bladder is never entirely empty coincides with

the well known clinical fact that the gall bladder is never found empty on abdominal exploration. The physiological importance of this fact may be explained by the suggestion of Boyden (12) that the capillary network of the rugæ of the gall bladder of the cat is greater in collapse than when pressed out by distension. He believes that the most rapid absorption takes place when the gall bladder is partially collapsed. It is during partial collapse of the gall bladder that the density of the shadow of a cholecystogram is maximum. The gall bladder is in this state of partial collapse for the longest period of time during its visibility by Roentgen ray.

We have noted that the shadow of the normal gall bladder varies in size, denoting that other factors than dilution or interchange of bile may play a part in the slow emptying of the gall bladder. The importance of changes of intraabdominal pressures, especially due to respiration, has been lately stressed by Winkelstein (13). The effect of respiration on the intravesical pressure may be demonstrated by placing a manometer in the gall bladder and closing the abdominal wall about it as was done in the anesthetized dog. There may be a variation as high as 60 mm. of bile during respiration, vomiting, and other changes of intraabdominal pressure. The changes of pressure in the ducts with respiration are not so large as those in the gall bladder. Bile in some instances may be seen to be expelled from the duodenal papilla with forced respiration. Furthermore, the shadow of a gall bladder in a dog may be decreased in size by deep palpation of the abdominal wall over the region of the gall bladder.

We believe with Boyden (12) that, as regards emptying, the gall bladder is largely a passive organ, except for the elasticity of its walls. It is influenced by pressure changes which we have described. The anatomy of the walls of the gall bladder are ideal for such an elastic or contractile mechanism. Its tonus varies in response to pressure changes. The smooth muscle fibers and connective tissue aid in preventing overdistension and prevent complete collapse of the walls. The distensible walls equalize extremes of pressure due to the variation of the rate of secretion of bile, to the variable state of contraction of the common duct sphincter, and to changes of intraabdominal pressure. The elasticity of the walls is also a mechanism for the expulsion of bile through the cystic duct. The maximal pressure which the wall

of the gall bladder can exert is difficult to determine *in situ*. It is probably great enough to expel bile from a distended gall bladder when the common duct sphincter is relaxed. Though the gall bladder has an elastic or contractile mechanism which exerts pressure on its contents until it has reached a minimum pressure, we have not been able to demonstrate an actively contracting movement due to its intrinsic musculature. Direct fluoroscopic examinations of visualized gall bladders have uniformly failed to show any evidence of a peristaltic wave; and more important still, direct electrical stimulation of the wall of the gall bladder of an anesthetized dog has never resulted in a contraction wave, although a similar stimulation of the intestine induced violent peristaltic contractions. This agrees with a previous observation made by Boyden (12) and Whitaker. Rhythmic changes of pressure of the content of the gall bladder have been recorded by us by various means in the anesthetized dog. However, no curves have been obtained from the gall bladder that have not been duplicated by a rubber bag of like size placed in the upper part of the abdominal cavity of the etherized dog. The rhythmic changes which were recorded, corresponded to changes of intraabdominal pressure largely due to respiration.

The same experiment as the preceding one was conducted on dogs after operation under ether anesthesia.

A rubber tube was connected to a small rubber bag which was placed in the fundus of the gall bladder. A similar bag was placed alongside the viscera of the upper abdomen. The rubber tubes were brought out of the abdominal wall through different incisions. The dogs were allowed to recover from their operations. The bags were distended with air and simultaneous readings made of the changes of pressure after feeding, alteration of position of the dog, etc. The records from each bag corresponded essentially. This experiment confirms the finding in the preceding one that the so called rhythmic contractions were independent of peristaltic or other active muscular contractions of the wall of the gall bladder.

There has not been an indication of an actively effective contraction of the gall bladder, such as a peristaltic movement of the intestine or evacuation of the urinary bladder, in the thousands of cholecystograms that have been made by this department in man and animals. All of the changes in size and shape of the shadows are referable to

an expansile organ. It is not necessary in any instance to explain the emptying of the gall bladder on the basis of an active muscular contraction of its wall.

Further proof of this rather passive reaction of the gall bladder is offered by the results obtained from an artificial gall bladder. A thin rubber bag the size of a gall bladder may be tied into a cystic duct of a dog by means of a short glass tube after cholecystectomy. If the animal is given a dose of sodium tetraiodophenolphthalein after the surgical operation, there will be a faint shadow of the artificial gall bladder visible in 4 hours. 16 hours later the shadow is larger and denser. The shadow may persist 6 or 8 days. The cycle of shadows produced by the artificial, rubber gall bladder is quite like that obtained from the normal gall bladder of the human, except that the shadows may not be so dense and that they usually persist over a longer period of time (Fig. 1, *a* and 1, *b*). The presence of the shadow seems to indicate that a forceful contraction due to a muscular action of the gall bladder wall is not necessary for the emptying or filling of the gall bladder. While the elastic or contractile mechanism present in the gall bladder is not necessary for its filling or emptying, it is probably the lack of this factor in the artificial gall bladder which delays its emptying. In this experiment, the increase in density of the shadow obviously cannot be due to concentration of the bile in the rubber bag but more probably must be due to a greater amount of iodine which has entered the bag through the cystic duct in its process of filling. In ordinary cholecystography the increase in density of the shadow which is observed is probably due in part to the factor of concentration by absorption of water. The shadow in the artificial gall bladder appears quite as promptly as in the normal gall bladder. The disappearance of the shadow must take place by washing out of the bile in the bag by fresh liver bile and by intraabdominal pressure. The pressure in the biliary tract is not sufficient to distend the rubber bag. Also, since the artificial gall bladder is not in apposition to the liver, changes in volume of that organ are not necessary for the filling or emptying of the gall bladder, although doubtlessly they are normally a factor. Changes of blood pressure in the normal gall bladder do not seem to be essential for its activity. This experiment, furthermore, casts considerable doubt on the contrary innerva-

tion of the gall bladder described by Meltzer (14). He has concluded that there is a coordinated action of relaxation of the sphincter of Oddi and contraction of the gall bladder. Lyon (15) has based the nonsurgical drainage of the gall tract upon this hypothesis.

CONCLUSIONS.

As a result of the control of the flow of bile into the duodenum largely by tonus and movements of the duodenum, bile intermittently enters the gall bladder where it is concentrated and undergoes other changes. The gall bladder empties itself of its content through the cystic duct (1) by the washing out of its contents by bile from the liver, (2) by the elasticity or contractile mechanism of its walls, and (3) by variations of intraabdominal pressure due to respiratory movements, contiguous organs, etc. A fourth manner of emptying of the gall bladder is by absorption of a portion of its content through its walls. The gall bladder is never entirely empty but tends to come to a state of partial collapse, when its contents are under minimum pressure. We have been unable to demonstrate rhythmic contractions of the gall bladder due to its musculature. If they are present they may aid but they are not essential for its emptying or filling. Experimentally, in the dog, a rubber bag which was substituted for the gall bladder functioned in a manner very similar to that of the normal gall bladder as shown by cholecystographic studies. The concentrating function, however, was absent.

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EXPLANATION OF PLATE 3.

FIG. 1, *a*. Arrows point to the shadow produced by an artificial, rubber gall bladder, 24 hours after intravenous injection of sodium tetraiodophenolphthalein. The small dense shadow within the shadow of the gall bladder is the glass tube which connected the bag with the cystic duct. The same tube shows in Fig. 1, *b*.

FIG. 1, *b*. Roentgenogram of the same dog 6 days later. The shadow of the artificial gall bladder has disappeared.

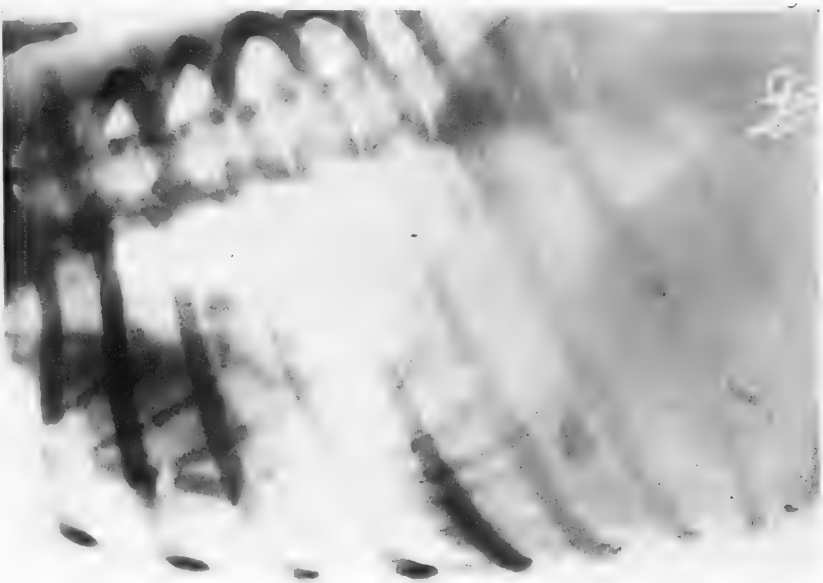

FIG. 1, *b*.

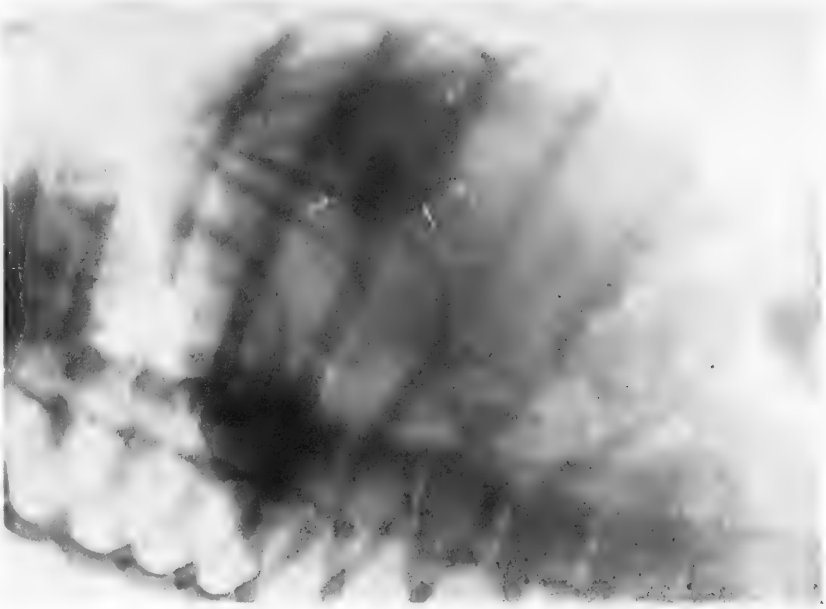

FIG. 1, *a*.

(Copher, Kodama, and Graham: Gall bladder.)

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THE RELATION OF THE RETICULO-ENDOTHELIAL SYSTEM TO THE BLOOD PLATELET COUNT.

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In studying the diminution in the number of blood platelets which is one of the most prominent factors in the pathogenesis of thrombocytopenic purpura, the author could not find positive evidence in the literature, of the cause of this thrombopenia. Many conflicting opinions were encountered, and these could be narrowed down to two basic ones. Frank and Glantzmann assume that the megacaryocytic apparatus is injured or inhibited from producing the normal number of platelets. Kaznelson and many others support the opposite view; namely, that there is an excessive thrombolytic function in the spleen and perhaps in other parts of the body, which causes a marked diminution in the number of platelets, not by interfering with their formation but by increasing their destruction. It has already been established that splenectomy in the experimental animal is followed by a rise in the number of platelets in the blood (Bedson). The spleen is therefore supposed to be the site of destruction of quantities of platelets, and its removal inhibits much of this destruction. This rise, Bedson has shown, is temporary, the return to normal being completed after about 1 month. The explanation advanced for the gradual return to normal is that the hyperactivity of other physiologically related tissue compensates for the loss occasioned by splenectomy. The hyperactivity is manifested by an increased platelet destruction and the hitherto increased number of platelets (as a result of splenectomy) is diminished to the normal level.

It has also been noted clinically that splenectomy in thrombocytopenic purpura is rapidly followed by a rise in the platelet count. This relatively high count soon becomes diminished however, the fall in

some cases almost reaching the low level before operation. Is it because of a similar compensatory activity of physiologically related tissue?

It becomes evident that investigation regarding the source of supply and the mechanism of destruction of the platelets is necessary to clear up the haze which surrounds these problems.

The investigations of Ribbert, Aschoff, Kiyono, Goldmann, and others have established the existence of a group of cells widely distributed throughout the interstitial and perivascular tissues of the body, possessing common morphological and physiological properties, which they have grouped as a tissue under the heading of the reticulo-endothelial system. To the activity of this tissue, of which the spleen contains a large amount, has been attributed the destruction of platelets, and the investigation along one line of the basis for such assertion furnishes the subject matter of this paper.

One of the most prominent characteristics of these cells is their propensity for absorbing and storing in granular form vital stains introduced into the blood in solution. They phagocytose these vital stains voraciously, and becoming gorged, they are rendered otherwise relatively inactive. This method of blocking the system has been widely utilized to determine indirectly the activities of these cells.

In this study of the possible relationship between the reticulo-endothelial system and platelet destruction, the blocking system was used, platelet counts being made before and after the inactivation and rather striking results were obtained.

Method.

Guinea pigs were used in all the experiments. Before proceeding with the main problem, the method of platelet counting was studied. It was found that widely different platelet counts could be obtained by the same observer, using the same method, the same pig, during the same hour, the difference depending upon the manner in which the drop of blood was obtained.

The exertion of pressure on the surrounding tissue after needle puncture, to secure a large enough drop of blood for the procedure, is uniformly productive of a count at considerable variance with that obtained when the blood flows freely from the injured surface. Sticking the ear lobe in the attempt to strike the analogue of the marginal vein of the rabbit almost always fails to give a sufficiently large sized drop, as does needle sticking elsewhere in the body. It

was found that the best way to secure a good drop was by incising the foot with a safety razor blade, after thorough cleansing.

Various methods of counting were experimented with. They may be classified as direct and indirect. The first is self-explanatory. The second is accomplished by the determination in a blood smear of the relation between the amount of platelets and the number of red cells, and then the number of red cells per c.mm. is counted, from which the actual number of platelets is estimated. Probably the best method of this group is that of Bedson. The direct method seemed preferable because the possibility of error is apparently less. Various staining solutions were used (Wright and Kinnicutt, Leak and Guy, Reese and Ecker, etc.), but it was finally decided that the source of error would be minimized by counting the platelets unstained, because in the process of staining, despite all caution and effort to prevent it, very frequently small particles of stain would

TABLE I.

Guinea Pig No.	Platelet count.					Weight.			
	Feb. 4	Feb. 5	Feb. 6	Feb. 7	Average for 4 days.	Feb. 4	Feb. 5	Feb. 6	Feb. 7
						gm.	gm.	gm.	gm.
1	286,000	364,000	292,000	310,000	313,000	458	404	413	402
2	336,000	338,000	288,000	314,000	319,000	446	396	407	412
3	251,000	234,000	174,000	262,000	230,500	467	430	450	511
4	324,000	216,000	386,000	364,000	322,500	547	506	514	531
5	306,000	324,000	362,000	260,000	313,000	439	425	445	451
6	336,000	316,000	330,000	332,000	328,500	428	413	410	400
Total average.....					304,416				

be precipitated and these simulated platelets, thus interfering with accurate counting. The method adopted was to use a red cell counting pipette, draw up to the 1 mark, add 3 per cent sodium citrate to the 101 mark, and then after thoroughly mixing to place a suitably sized drop in a counting chamber, allow to settle for 15 minutes, count the platelets in 400 squares, and multiply the result by 1000. Because of the personal equation variation all counts were made by the same observer.

EXPERIMENTAL.

Six guinea pigs of approximately the same size were obtained from one source. They were segregated and watched for 4 days for signs of ill health. None being manifest, daily platelet counts were made on each one, over a period of 4 days. The counts ranged from a low level of 174,000 to a high level of 386,000. The average count for each pig was established as was also the total average. The latter was 304,416. (See Table I.)

TABLE II.

February 8. Injection of 5 cc. of ½ per cent solution of trypan blue in each pig.
February 12. 2nd injection of 5 cc. of ½ per cent solution of trypan blue.

Guinea Pig No.	Platelet count.									Weight.							
	Feb. 10	Feb. 11	Feb. 12, 2nd injection.	Feb. 16	Feb. 17	Feb. 18	Feb. 19	Feb. 20	Average for 4 days beginning Feb. 16.	Feb. 10	Feb. 11	Feb. 12, 2nd injection.	Feb. 16	Feb. 17	Feb. 18	Feb. 19	Feb. 20
										gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	372,000	405,000	424,000	2,004,000	1,500,000	808,000	480,000	848,000	1,198,000	455	448	462	457	443	483	463	465
2	418,000	396,000	414,000	568,000	708,000	948,000	864,000	820,000	772,000	420	428	415	381	379	376	361	368
3	205,000	207,000	382,000	1,732,000	1,206,000	1,420,000	880,000	910,000	1,309,500	419	430	412	386	406	429	425	430
4	402,000	256,000	476,000	526,000	1,140,000	840,000	514,000	536,000	755,000	495	485	483	463	512	507	496	443
5	231,000	290,000	382,000	Died.						369	402	345					
6	372,000	462,000	392,000	1,360,000	930,000	464,000	744,000	688,000	824,500	425	423	429	418	447	480	460	462
Total average.....									984,000								

Then 5 cc. of a ½ per cent solution of trypan blue in freshly distilled water, sterilized by boiling, was injected subcutaneously into each animal. The trypan blue used in these experiments was obtained from the A. H. Thomas Co., of Philadelphia, some products of other derivation having been found highly toxic and therefore undesirable. Daily counts by the same technique were continued and the injections repeated 4 days later. On that day Pig 67 died. 9 days after the first trypan blue injection the count level had so changed that now the low level was 708,000 while the high reached 1,500,000. The total average for a period of 4 days, begun 8 days after the first trypan blue injection was 984,000, showing an increase over the total average, before injection, of 685,000. (See Table II.)

Very frequently during the experiment, check was made upon the platelet count and the degree of error was found to be very slight, being less than 5 per

TABLE III.

February 23. Injection of 5 cc. of ½ per cent solution of trypan blue in each pig.

Guinea Pig No.	Platelet count.						Weight.					
	Feb. 23	Feb. 24	Feb. 25	Feb. 26	Feb. 27	Mar. 1	Feb. 23	Feb. 24	Feb. 25	Feb. 26	Feb. 27	Mar. 1
							gm.	gm.	gm.	gm.	gm.	gm.
1	708,000	844,000	694,000	728,000	676,000	576,000	483	489	475	470	468	482
2	694,000	468,000	512,000	460,000	402,000	364,000	380	303*	342*	320*	331*	343
3	776,000	620,000	808,000	796,000	622,000	760,000	474	480	465	470	465	484
4	640,000	604,000	802,000	940,000	776,000	762,000	507	540	517	518	522	540
6	984,000	864,000	636,000	662,000	548,000	608,000	487	478	482	497	511	536

* Diarrhea.

cent. Bedson and Zilva rate the possibility of error in counting platelets by their method at about 10 per cent. The estimated degree of possible error in this work bears out the previously expressed view that the direct method of counting is by far more desirable. However, in the light of the marked differences in counts obtained before and after the blockade, it is readily seen that even a much more generous allowance for error would not vitiate the results.

Two control animals, of similar stock, cared for under similar conditions, received injection of 5 cc. of distilled water at the same time that the test animals received the trypan blue injections. Those before injection showed a total average of 288,000 whilst the total average for the 4 days beginning 8 days after the first injection of distilled water was 278,000. Thus the rise in platelets in the other series is undoubtedly related to the injection of trypan blue.

The platelet counts were continued as shown in Table III. Apparently after the initial high excursion, there is a slight recoil and then a slow diminution in their numbers. (See Table III.)

Thus, after injection of trypan blue the platelet count shows a marked rise, the total average increase equalling approximately 300 per cent. This is followed by a sharp recoil and then a more gradual decline, despite another injection of trypan blue to continue the saturation. A graphic representation of the variation is given in Chart 1.

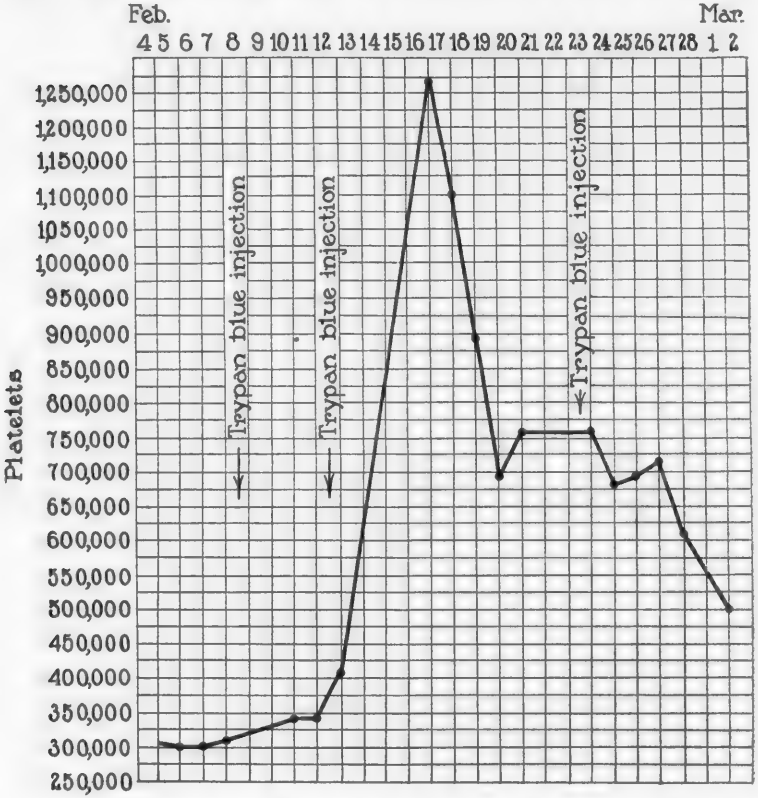

CHART 1. Graphic representation of the daily average platelet count of all the pigs used in this experiment.

DISCUSSION.

In analyzing the results of the experiment, it soon becomes apparent that many different conclusions might be drawn regarding the rela-

tionship of the reticulo-endothelial system to platelet destruction. Comparing the average counts before and after blockade, it would appear that the saturation interferes with the phagocytosis of platelets to a considerable degree. After a study of the graph however, the question as to what causes the sharp decline immediately suggests itself.

The later literature dealing with reticulo-endothelial system blockade by dyes and colloids supports the contentions of the earlier workers that the cells can be so overburdened as to have their activities paralyzed. The reticulo-endothelial cells have also been considered as the agents by which blood destruction is accomplished, chiefly because phagocytosis of red and white cells and platelets by the macrophages has been repeatedly noted. Such phagocytosis is especially prominent in pathological conditions. Asch found it prominent in the spleen in pernicious anemia. Seidelin reported it in malaria. Pool and Stillman recorded phagocytosis of platelets in the spleen in thrombocytopenic purpura. Enormous phagocytosis of erythrocytes occurs in birds poisoned by arsine. But even in normal healthy mammals such phagocytosis has long been recognized to occur in the spleen. Assuming then that the trypan blue completely blocked the reticulo-endothelial system and that phagocytosis is the only means by which platelets are destroyed, it would be expected that the platelets would continue to increase *ad infinitum* provided there was no inhibition of production.

It is doubtful however, whether all the reticulo-endothelial cells can be so completely stuffed as to be unable to function. As Rich says, "One might well expect the cells to be unable to live under such conditions." The blockade, and the inactivity, must be relative.

It is also doubtful whether phagocytosis is the only method of destroying platelets. In the case of the erythrocytes the evidence as reviewed by Rous decidedly favors the view that phagocytosis is not the only means for their destruction. Rous and Robertson have shown, for instance, that the cat, an animal with a very active hematopoietic system, has very few macrophages. Indeed, whether the cells which do undergo phagocytosis are primarily devoured by the macrophages, or whether they are first rendered inert and then picked up and metabolized merely as any other foreign bodies are, is still

open to question. It should be remembered that platelets are a very unstable quantity in the blood. The wide range of variation in the count under apparently normal conditions, the ease and rapidity with which they can be driven from the circulation by the injection of snake venom (Flexner), albumose (Gley), the rapidity with which they return to the circulation in severe cases of purpura hæmorrhagica after splenectomy, bespeak a very labile regulatory apparatus. And the side which deals with platelet production must not be overlooked.

The graph shows that after the initial marked rise and subsequent sharp decline, there is a steady diminution in the platelets despite the reinforcement of the blockade by another trypan blue injection. The possible explanations which suggest themselves are: (1) that the blockade is no longer as complete, despite the reinforcement, and that phagocytosis is now more active; (2) that in time some other mechanism assumes the added burden of platelet destruction as a compensatory reaction; (3) that the original trypan injection, while it blockaded the reticulo-endothelial system, sharply stimulated the platelet-producing mechanism to greater activity as manifested by the sudden and great increase in platelets, and subsequent injections failed because of acquired tolerance.

Achard and Aynaud report marked diminution in the platelet count after intravenous injection of electrargol. And yet many workers have used electrargol to block the reticulo-endothelial system. This is apparently a contradiction if blocking the system should cause a rise in the platelets. It would favor the idea that there is either some very active mechanism for platelet destruction beside phagocytosis, or that this particular agent caused inhibition of platelet production.

In another series of experiments undertaken to determine the effect of trypan blue blockade on the development of immune bodies, it was found that after the blockade had been established the injection of a small quantity of washed sheep red blood cells produced a fall from 1,000,000 platelets to 200,000 in 12 hours. This is also suggestive of other means of diminution in numbers besides phagocytosis.

It becomes quite evident that at this stage in our knowledge of the life cycle of the platelet it is far more important to accumulate data than to attempt to evolve hypotheses and construct theories, and that proper interpretation of the phenomena is dependent on the acquisition of more facts.

SUMMARY.

1. So called blockade of the reticulo-endothelial system results in a marked increase in the platelet count.

2. The count rapidly becomes very high, shows a sharp partial recoil and then a gradual diminution even though the blockade be continued.

3. The mechanism regulating the number of platelets is a complicated one.

4. Apparently phagocytosis by cells of the reticulo-endothelial system is not the only means by which blood platelets are destroyed.

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RELATION BETWEEN ORGAN WEIGHTS AND OBSCURE LESIONS IN APPARENTLY NORMAL RABBITS.

Second Paper.

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An analysis of the results obtained from a study of organ weights of normal rabbits with reference to effects that might be produced by obscure lesions led to the conclusion (1) that while any abnormality might give rise to some change in the mass and mass relationships of organs, the magnitude of the change was of such an order that it was barely perceptible until the abnormality was sufficiently pronounced to be recognized by signs and symptoms of disease or by physical deterioration. In apparently healthy and vigorous rabbits it appeared that only those with extensive lesions were apt to show differences in the mean weights of organs that are of any material significance from a statistical point of view. It was thought, however, that even the small differences noted suggested a tendency to the occurrence of progressive changes in weight which bore some relation to the reaction of the animal to disease as indicated by the extent of the lesions present. In order to obtain further information concerning changes in organ weights that may occur in association with obscure disease conditions, an analysis of the results of a second series of weight determinations was undertaken. The object of this paper is to report the results of this investigation, which will include a consideration of the relation between organ weight and the activity of the lesions as well as the extent of the lesions found on postmortem examination.

Methods and Material.

The results to be reported are based on the examination of 295 male rabbits killed between July 1, 1924, and July 1, 1925, representing Series II of the normal rabbits included in the preceding paper of this group (2).

TABLE I.

Results for Body and Organ Weights According to Groups.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Gross body weight.	II	295	2257.8	2225	1600	3200	306.30	206.60	13.56
	1	138	2253.4	2225	1600	3200	311.90	210.40	13.84
	2	80	2308.1	2275	1775	3100	294.30	198.50	12.74
	A	16	2385.9	2287.5	1950	3025	349.20	235.50	14.63
	I	64	2288.6	2275	1775	3100	275.60	185.90	12.04
	3	51	2241.6	2150	1650	3075	296.50	199.90	13.20
	A	19	2252.6	2175	1800	2775	293.60	198.03	13.03
	I	32	2235.0	2150	1650	3075	300.70	202.80	13.45
	4	26	2159.6	2075	1625	2925	304.40	205.30	14.09
	A	13	2071.0	2025	1775	2500	221.40	149.30	10.69
	I	13	2248.1	2200	1625	2925	348.20	234.90	15.40
Gastrointestinal mass. Actual.	II	295	424.4	423	195	740	79.30	53.49	18.68
	1	138	421.3	420	245	740	79.24	53.45	18.81
	2	80	437.8	425	270	695	82.45	55.61	18.83
	A	16	451.6	450	295	615	86.68	58.47	19.19
	I	64	434.4	425	270	695	81.07	54.68	18.66
	3	51	427.6	440	195	585	76.40	51.53	17.86
	A	19	411.8	435	195	585	101.80	68.66	24.72
	I	32	437.0	440	280	565	54.40	36.70	12.44
	4	26	391.9	375	270	580	65.40	44.11	16.60
	A	13	396.5	380	350	495	42.80	28.86	10.79
	I	13	387.3	360	270	580	84.20	56.79	21.74
Relative.	II	295	235.2	230.00	118.0	452.0	49.90	33.70	21.21
	1	138	234.5	229.75	141.5	446.0	49.90	33.70	21.27

	2	80	237.2	229.25	128.3	452.0	50.30	33.90	21.20
	A	16	237.5	230.00	166.0	344.0	46.20	31.20	19.45
	I	64	237.2	229.50	128.3	452.0	51.40	34.70	21.66
	3	51	240.2	241.00	117.0	374.0	53.50	36.10	22.27
	A	19	225.8	230.00	117.0	351.0	54.90	37.03	24.31
	I	32	248.8	246.00	156.0	374.0	50.80	34.30	20.41
	4	26	223.9	220.00	171.0	357.5	34.60	23.30	15.40
	A	13	207.5	199.00	171.0	313.0	25.40	17.10	12.24
	I	13	240.4	234.00	177.5	357.5	38.10	25.70	15.80
Net body weight.	II	295	1833.1	1800.0	1210	2660	273.00	184.10	14.89
	1	138	1832.0	1805.0	1290	2630	280.03	188.80	15.29
	2	80	1869.6	1842.5	1370	2545	258.92	174.64	13.84
	A	16	1933.1	1910.0	1565	2483	295.60	199.40	15.29
	I	64	1853.8	1835.0	1370	2545	227.95	153.75	12.29
	3	51	1812.8	1715.0	1210	2660	273.70	184.60	15.09
	A	19	1830.5	1790.0	1490	2350	236.10	159.30	12.89
	I	32	1801.1	1702.5	1210	2660	293.40	197.90	16.29
	4	26	1768.8	1735.0	1355	2500	276.70	186.60	15.60
	A	13	1674.6	1580.0	1360	2135	215.50	145.40	12.86
	I	13	1863.1	1815.0	1355	2500	285.20	192.40	15.30
Heart.	II	295	5.31	5.22	3.30	9.02	0.93	0.627	17.51
Actual.	1	138	5.305	5.40	3.30	9.02	0.908	0.612	17.09
	2	80	5.30	5.19	3.45	8.14	0.902	0.608	17.01
	A	16	5.57	5.71	4.00	8.14	1.05	0.71	18.85
	I	64	5.24	5.15	3.45	8.05	0.874	0.59	16.67
	3	51	5.34	5.30	3.47	7.43	0.896	0.604	16.77
	A	19	5.39	5.30	3.55	7.40	0.943	0.636	17.49
	I	32	5.31	5.31	3.47	7.43	0.867	0.585	16.32
	4	26	5.34	5.29	3.38	7.63	1.13	0.76	21.16
	A	13	5.32	5.05	3.79	7.50	1.066	0.719	20.00
	I	13	5.36	5.45	3.38	7.63	1.19	0.803	22.21

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Heart.	II	295	2.90	2.88	2.04	4.36	0.339	0.229	11.68
Relative.	1	138	2.89	2.86	2.27	4.04	0.336	0.227	11.62
	2	80	2.86	2.83	2.04	4.36	0.372	0.251	13.00
	A	16	2.88	2.85	2.34	3.27	0.214	0.144	7.43
	I	64	2.85	2.82	2.04	4.36	0.402	0.271	14.21
	3	51	2.95	2.94	2.15	3.57	0.277	0.187	9.38
	A	19	2.94	2.96	2.15	3.57	0.348	0.235	11.83
	I	32	2.95	2.90	2.55	3.54	0.224	0.151	7.59
	4	26	3.01	3.05	2.44	3.84	0.336	0.227	11.16
	A	13	3.16	3.05	2.85	3.84	0.299	0.202	9.46
	I	13	2.85	2.94	2.44	3.29	0.305	0.206	10.70
Liver.	II	295	87.52	85.00	47.00	185.00	19.29	13.01	22.04
Actual	1	138	87.05	85.00	47.00	153.00	18.46	12.45	21.19
	2	80	88.26	86.00	57.00	152.00	19.21	12.96	21.90
	A	16	96.81	101.00	65.00	116.00	12.25	8.26	12.66
	I	64	86.12	80.00	57.00	152.00	20.10	13.56	23.34
	3	51	85.54	80.00	50.00	158.00	18.50	12.48	21.60
	A	19	84.10	85.00	55.00	117.00	17.60	11.87	20.92
	I	32	86.40	80.00	50.00	158.00	19.80	13.40	22.90
	4	26	94.96	92.00	63.00	185.00	23.07	15.56	24.29
	A	13	98.00	90.00	73.00	185.00	26.80	18.10	27.34
	I	13	91.92	92.00	63.00	120.00	18.70	12.60	20.34
Relative.	II	295	48.47	46.70	25.80	117.00	12.10	8.16	24.96
	1	138	48.32	46.60	25.80	92.20	10.86	7.33	22.47

	2	80	47.73	45.80	27.80	88.30	11.50	7.76	24.10
	A	16	51.37	49.30	36.90	75.80	10.60	7.15	20.60
	I	64	46.81	44.80	27.80	88.30	11.40	7.69	24.30
	3	51	46.88	45.90	29.50	95.30	11.28	7.61	24.06
	A	19	46.22	47.30	32.40	69.00	8.82	5.95	19.08
	I	32	47.27	45.10	29.50	95.30	12.49	8.42	26.42
	4	26	54.80	51.70	36.90	117.00	15.39	10.38	28.08
	A	13	59.80	59.00	36.90	117.00	19.10	12.88	31.93
	I	13	49.80	47.10	37.30	65.20	7.70	5.19	15.46
Kidneys.	II	294	13.22	13.05	8.33	20.00	2.057	1.39	15.55
Actual.	1	137	13.06	13.09	8.88	20.00	2.028	1.367	15.52
	2	80	13.29	13.02	10.00	19.40	2.03	1.37	15.27
	A	16	13.40	12.85	10.00	19.40	2.45	1.65	18.28
	I	64	13.27	13.07	10.01	18.90	1.91	1.29	14.39
	3	51	13.13	13.05	8.33	17.40	1.82	1.23	13.86
	A	19	13.11	12.96	9.25	15.40	1.61	1.09	12.28
	I	32	13.15	13.05	8.33	17.40	1.94	1.31	14.75
	4	26	14.00	13.52	9.02	19.97	2.54	1.71	18.14
	A	13	13.60	12.92	11.05	17.56	2.02	1.36	14.85
	I	13	14.40	13.70	9.02	19.97	2.92	1.97	20.27
Relative.	II	294	7.32	7.23	4.29	12.80	1.212	0.817	16.55
	1	137	7.23	7.07	4.29	12.80	1.15	0.77	15.90
	2	80	7.23	7.23	5.20	10.39	1.07	0.72	14.80
	A	16	6.96	7.10	5.64	8.27	0.84	0.567	12.07
	I	64	7.30	7.24	5.20	10.39	1.17	0.789	16.03
	3	51	7.34	7.23	4.91	11.52	1.20	0.81	16.35
	A	19	7.22	7.34	4.91	8.62	0.964	0.65	13.35
	I	32	7.42	7.13	5.35	11.52	1.32	0.89	17.78
	4	26	8.00	7.94	5.25	10.54	1.40	0.944	17.50
	A	13	8.18	7.94	6.12	10.54	1.25	0.843	15.28
	I	13	7.81	8.10	5.25	10.10	1.53	1.03	19.59

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Spleen.	II	295	1.065	0.985	0.160	5.050	0.549	0.370	51.54
Actual.	1	138	0.973	0.860	0.240	4.480	0.601	0.450	61.76
	2	80	1.060	1.025	0.320	2.370	0.448	0.302	42.26
	A	16	1.111	1.005	0.530	1.940	0.411	0.277	36.99
	I	64	1.048	1.013	0.320	2.370	0.457	0.308	43.60
	3	51	1.192	1.150	0.160	5.050	0.703	0.474	58.97
	A	19	1.230	1.150	0.160	5.050	0.969	0.654	78.78
	I	32	1.169	1.113	0.525	2.650	0.481	0.324	41.14
	4	26	1.315	1.192	0.600	2.685	0.533	0.359	40.53
	A	13	1.325	1.220	0.600	2.135	0.420	0.283	31.69
	I	13	1.307	1.185	0.660	2.685	0.620	0.418	47.14
Relative.	II	295	0.587	0.528	0.107	2.930	0.326	0.220	55.53
	1	138	0.520	0.463	0.140	2.140	0.3004	0.203	57.76
	2	80	0.572	0.549	0.168	1.480	0.246	0.166	43.01
	A	16	0.582	0.574	0.262	0.964	0.213	0.144	36.60
	I	64	0.570	0.539	0.168	1.480	0.254	0.171	44.50
	3	51	0.670	0.631	0.107	2.930	0.414	0.279	61.79
	A	19	0.679	0.545	0.107	2.930	0.568	0.383	83.65
	I	32	0.665	0.653	0.287	1.620	0.292	0.197	43.90
	4	26	0.751	0.722	0.355	1.440	0.302	0.204	40.21
	A	13	0.785	0.722	0.441	1.282	0.196	0.132	24.96
	I	13	0.717	0.544	0.355	1.440	0.352	0.237	49.09
Thymus.	II	295	2.365	2.250	0.37	6.30	0.842	0.568	35.67
Actual.	1	135	2.450	2.350	0.83	6.30	0.848	0.572	34.61

	2	80	2.406	2.325	0.95	5.65	0.779	0.525	32.37
	A	16	2.694	2.530	0.95	5.65	1.133	0.764	42.05
	I	64	2.334	2.260	0.97	4.10	0.643	0.434	27.54
	3	51	2.151	2.180	0.37	4.12	0.870	0.587	40.44
	A	19	1.918	1.800	0.37	3.85	0.895	0.604	46.66
	I	32	2.289	2.250	0.73	4.12	0.848	0.572	37.04
	4	26	2.149	2.050	0.63	3.50	0.806	0.543	37.50
	A	13	2.318	2.325	0.63	3.50	0.862	0.581	37.18
	I	13	1.980	1.710	1.22	3.50	0.712	0.480	35.95
Relative.	II	295	1.304	1.269	0.248	3.315	0.464	0.313	35.58
	I	138	1.356	1.317	0.471	3.315	0.477	0.322	35.17
	2	80	1.298	1.291	0.566	2.817	0.407	0.274	31.35
	A	16	1.401	1.392	0.578	2.817	0.551	0.372	39.32
	I	64	1.272	1.269	0.566	1.995	0.358	0.241	28.14
	3	51	1.199	1.195	0.248	2.430	0.496	0.335	41.36
	A	19	1.046	1.045	0.248	1.930	0.476	0.321	45.50
	I	32	1.291	1.243	0.388	2.430	0.485	0.327	37.56
	4	26	1.258	1.187	0.315	2.290	0.536	0.362	42.60
	A	13	1.453	1.525	0.315	2.292	0.623	0.420	42.87
	I	13	1.062	0.964	0.600	1.790	0.333	0.225	31.35
Testicles.									
Actual.	II	294	4.50	4.48	0.92	9.01	1.415	0.954	31.44
	1	137	4.38	4.35	1.36	8.69	1.440	0.971	34.61
	2	80	4.76	4.63	1.36	8.05	1.360	0.924	28.60
	A	16	4.80	4.74	2.50	7.05	1.350	0.910	28.10
	I	64	4.74	4.63	1.36	8.05	1.360	0.920	28.60
	3	51	4.51	4.70	0.92	8.32	1.430	0.965	31.70
	A	19	4.65	4.90	2.92	8.32	1.180	0.796	25.37
	I	32	4.42	4.60	0.92	7.38	1.560	1.050	35.29
	4	26	4.39	4.48	1.69	9.01	1.430	0.965	32.57
	A	13	4.45	4.45	1.69	9.01	1.640	1.100	36.85
	I	13	4.34	4.60	1.86	5.80	1.070	0.720	24.65

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Testicles.	II	294	2.45	2.42	0.562	4.220	0.650	0.440	26.53
Relative.	I	137	2.37	2.37	0.902	4.160	0.640	0.432	27.00
	2	80	2.54	2.50	0.990	4.220	0.610	0.411	24.01
	A	16	2.47	2.53	1.260	3.335	0.440	0.300	17.80
	I	64	2.57	2.48	0.990	4.220	0.639	0.431	24.80
	3	51	2.47	2.43	0.562	4.170	0.680	0.460	27.53
	A	19	2.54	2.28	1.740	4.170	0.540	0.360	21.25
	I	32	2.43	2.47	0.562	3.910	0.750	0.500	30.86
	4	26	2.45	2.46	1.250	4.220	0.650	0.440	26.53
	A	13	2.57	2.73	1.250	4.220	0.770	0.520	29.96
	I	13	2.32	2.44	1.380	3.020	0.480	0.324	20.69
Brain.	II	295	9.11	9.20	5.80	11.33	0.687	0.463	7.54
Actual.	I	138	9.08	9.15	5.80	11.30	0.692	0.467	7.62
	2	80	9.19	9.30	7.43	10.80	0.630	0.425	6.80
	A	16	9.07	9.06	7.57	10.80	0.740	0.500	8.16
	I	64	9.22	9.32	7.43	10.63	0.586	0.395	6.35
	3	51	9.16	9.25	7.94	11.33	0.604	0.407	6.59
	A	19	9.37	9.35	8.10	11.33	0.649	0.437	6.93
	I	32	9.04	9.18	7.94	10.45	0.538	0.363	5.95
	4	26	8.88	8.90	8.00	9.82	0.448	0.302	5.04
	A	13	8.77	8.85	8.00	9.60	0.420	0.283	4.79
	I	13	8.99	8.95	8.00	9.82	0.478	0.322	5.32
Relative.	II	295	5.06	5.01	3.45	7.08	0.678	0.457	13.39
	1	138	5.05	4.98	3.45	7.08	0.6501	0.438	12.87
	2	80	5.03	4.99	3.67	6.84	0.668	0.451	13.28
	A	16	4.81	4.65	3.77	5.76	0.625	0.422	12.99
	I	64	5.09	5.04	3.67	6.84	0.670	0.452	13.16

	3	51	5.14	5.22	3.66	7.02	0.648	0.437	12.60
	A	19	5.17	5.13	4.20	6.21	0.510	0.340	9.86
	I	32	5.12	5.27	3.66	7.02	0.716	0.483	13.98
	4	26	5.11	4.99	3.93	6.69	0.679	0.455	7.52
	A	13	5.31	5.32	4.33	6.69	0.630	0.425	11.86
	I	13	4.91	4.85	3.93	6.60	0.620	0.418	12.62
Thyroid. Actual.	II	295	0.2356	0.210	0.085	1.730	0.1326	0.08944	56.28
	1	138	0.2175	0.200	0.090	0.545	0.08285	0.05588	38.09
	2	80	0.2603	0.210	0.100	1.730	0.2061	0.13901	79.17
		(79)	(0.24170)		(0.090)	(0.935)	(0.1238)	(0.0835)	(51.22)
	A	16	0.2183	0.2175	0.105	0.325	0.0602	0.0406	27.57
	I	64	0.2709	0.209	0.100	1.730	0.2274	0.15338	83.94
		(63)	(0.2477)		(0.100)	(0.935)	(0.1349)	(0.09099)	(54.46)
	3	51	0.23645	0.220	0.135	0.480	0.0824	0.05558	34.82
	A	19	0.23426	0.212	0.135	0.512	0.0922	0.06219	39.36
	I	32	0.23775	0.2225	0.140	0.480	0.07606	0.05133	31.98
	4	26	0.2546	0.250	0.085	0.775	0.12202	0.0823	47.92
	A	13	0.2406	0.245	0.155	0.340	0.05209	0.03513	21.65
	I	13	0.2686	0.250	0.085	0.775	0.16310	0.11001	60.72
Relative	II	295	0.13030	0.1149	0.0481	1.230	0.08119	0.05476	62.31
	1	138	0.1199	0.1084	0.0569	0.283	0.04611	0.03111	38.46
	2	80	0.14403	0.11435	0.0562	1.230	0.1371	0.09247	95.21
		(79)	(0.13030)		(0.0562)	(0.392)	(0.0626)	(0.0422)	(48.04)
	A	16	0.1131	0.10745	0.0686	0.1968	0.0291	0.01963	25.72
	I	64	0.1518	0.1174	0.0562	1.230	0.1515	0.10219	99.80
		(63)	(0.1347)		(0.0562)	(0.392)	(0.0678)	(0.0457)	(50.33)
	3	51	0.13069	0.1215	0.0798	0.3135	0.0415	0.02799	31.75
	A	19	0.12685	0.1215	0.0798	0.218	0.0398	0.02685	31.36
	I	32	0.13297	0.12025	0.0843	0.3135	0.0423	0.02853	31.80
	4	26	0.1428	0.1383	0.0481	0.310	0.0517	0.03487	36.20
	A	13	0.1492	0.141	0.0994	0.2092	0.0369	0.02489	24.73
	I	13	0.1397	0.136	0.0481	0.3100	0.0631	0.04256	45.16

TABLE I—*Continued*.

Organ	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Parathyroids.	II	292	0.0132	0.012	0.003	0.035	0.00551	0.00372	41.74
Actual.	1	137	0.01299	0.012	0.004	0.030	0.00502	0.00339	38.60
	2	79	0.01325	0.012	0.003	0.035	0.005403	0.00364	40.77
	A	16	0.01337	0.012	0.006	0.025	0.00478	0.00322	35.75
	I	63	0.01322	0.012	0.003	0.035	0.00555	0.00374	41.98
	3	50	0.01384	0.012	0.005	0.027	0.00522	0.00352	37.71
	A	19	0.01442	0.012	0.010	0.025	0.00515	0.00347	35.71
	I	31	0.01348	0.012	0.005	0.027	0.00524	0.00353	38.81
	4	26	0.01388	0.013	0.006	0.033	0.00616	0.00415	44.38
	A	13	0.01353	0.012	0.006	0.033	0.00659	0.00444	48.70
	I	13	0.01424	0.013	0.007	0.025	0.00565	0.00381	39.67
Relative.	II	292	0.00730	0.00667	0.00178	0.01850	0.00289	0.00195	39.60
	1	137	0.007094	0.00639	0.00266	0.01366	0.00269	0.001814	37.94
	2	79	0.00722	0.00656	0.00178	0.01850	0.00306	0.00206	42.38
	A	16	0.00696	0.00598	0.00383	0.01467	0.00265	0.00179	38.07
	I	63	0.00729	0.00667	0.00178	0.01850	0.00315	0.00212	43.20
	3	50	0.0077	0.00639	0.00306	0.01515	0.00295	0.00199	38.31
	A	19	0.0079	0.00725	0.00482	0.01501	0.0027	0.00182	34.17
	I	31	0.00757	0.00636	0.00306	0.01515	0.00309	0.00208	40.81
	4	26	0.00785	0.00758	0.00382	0.01545	0.00311	0.00210	39.61
	A	13	0.00793	0.00735	0.00382	0.01545	0.00301	0.00203	37.95
	I	13	0.00776	0.00823	0.00409	0.01454	0.00319	0.00215	41.10
Hypophysis.	II	294	0.0283	0.028	0.015	0.050	0.00553	0.00373	19.55
Actual.	1	138	0.028	0.027	0.015	0.050	0.00623	0.0042	22.24

	2	80	0.0295	0.0295	0.015	0.040	0.00535	0.00361	18.11
	A	16	0.0311	0.030	0.022	0.050	0.00654	0.00441	21.01
	I	64	0.02914	0.0285	0.015	0.040	0.00492	0.00235	16.88
	3	50	0.02782	0.028	0.020	0.040	0.00418	0.00282	15.02
	A	19	0.02742	0.028	0.022	0.033	0.00331	0.00223	12.08
	I	31	0.02806	0.028	0.020	0.040	0.00461	0.00311	16.42
	4	26	0.02715	0.0255	0.020	0.035	0.00383	0.00258	14.10
	A	13	0.02707	0.027	0.020	0.035	0.00417	0.00281	15.40
	I	13	0.02723	0.025	0.023	0.035	0.00352	0.00237	12.92
Relative.	II	294	0.01566	0.01535	0.0078	0.0352	0.00322	0.00217	20.56
	1	138	0.0155	0.01505	0.0078	0.0352	0.00351	0.00237	22.64
	2	80	0.01606	0.01577	0.00944	0.0247	0.00294	0.00198	18.30
	A	16	0.0164	0.01605	0.01054	0.0255	0.00479	0.00323	29.20
	I	64	0.01598	0.01587	0.00944	0.0247	0.00274	0.00184	17.14
	3	50	0.01557	0.01591	0.01005	0.0244	0.00292	0.00197	18.75
	A	19	0.01522	0.01565	0.01005	0.01942	0.00262	0.00177	17.21
	I	31	0.01579	0.01591	0.01096	0.0244	0.00307	0.00207	19.43
	4	26	0.01567	0.01614	0.00960	0.0235	0.00304	0.00205	19.40
	A	13	0.01640	0.01655	0.01143	0.0235	0.00318	0.00215	19.39
	I	13	0.01495	0.01580	0.0096	0.01845	0.00389	0.00262	26.02
Suprarenals.	II	295	0.3802	0.350	0.138	1.050	0.1646	0.11102	43.29
Actual.	1	138	0.3571	0.3335	0.145	0.987	0.14206	0.09582	39.78
	2	80	0.39015	0.350	0.160	0.885	0.1612	0.10873	41.31
	A	16	0.40593	0.3875	0.240	0.770	0.1416	0.09551	34.93
	I	64	0.3862	0.3375	0.160	0.885	0.1655	0.11163	42.85
	3	51	0.40245	0.360	0.165	0.900	0.1717	0.11581	42.65
	A	19	0.37157	0.330	0.165	0.900	0.1775	0.11972	47.76
	I	32	0.42078	0.3775	0.200	0.720	0.1683	0.11352	39.99
	4	26	0.4284	0.3575	0.138	1.050	0.2388	0.16107	55.74
	A	13	0.35561	0.310	0.138	0.760	0.1736	0.11709	48.81
	I	13	0.50115	0.400	0.200	1.050	0.3876	0.26144	77.34

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Suprarenals.	II	295	0.2078	0.185	0.0803	0.572	0.08460	0.05706	40.71
Relative.	1	138	0.19559	0.17735	0.0803	0.489	0.06938	0.04679	35.47
	2	80	0.21218	0.1882	0.0922	0.572	0.0903	0.06091	42.55
	A	16	0.21657	0.1810	0.1321	0.429	0.0881	0.05942	40.67
	I	64	0.21108	0.1928	0.0922	0.572	0.0911	0.06145	43.15
	3	51	0.22049	0.2182	0.0997	0.4425	0.0777	0.0524	35.23
	A	19	0.20284	0.1565	0.0997	0.4425	0.09038	0.06096	44.56
	I	32	0.23097	0.23165	0.1108	0.4225	0.0777	0.05241	33.63
	4	26	0.238	0.1945	0.088	0.564	0.1217	0.08209	51.13
	A	13	0.2084	0.177	0.088	0.3651	0.0872	0.05882	41.84
	I	13	0.2675	0.225	0.144	0.564	0.1425	0.09612	53.27
Pineal.	II	292	0.0154	0.015	0.005	0.040	0.00501	0.00338	32.53
Actual.	1	137	0.01561	0.015	0.006	0.035	0.00485	0.00327	31.07
	2	78	0.01561	0.015	0.005	0.035	0.00491	0.00331	31.47
	A	16	0.01562	0.017	0.008	0.030	0.0053	0.00358	33.93
	I	62	0.01561	0.015	0.005	0.035	0.00483	0.00326	30.94
	3	51	0.01574	0.015	0.005	0.040	0.00552	0.00372	35.06
	A	19	0.01557	0.015	0.005	0.020	0.00341	0.0023	21.90
	I	32	0.01584	0.015	0.009	0.040	0.00648	0.00437	40.90
	4	26	0.01365	0.013	0.005	0.025	0.00461	0.00311	33.77
	A	13	0.01323	0.014	0.005	0.018	0.00304	0.00205	22.97
	I	13	0.01407	0.012	0.007	0.025	0.00574	0.00387	40.79
Relative.	II	292	0.00853	0.00836	0.00228	0.0247	0.0029	0.00196	33.99
	1	137	0.00764	0.008	0.00369	0.01775	0.00276	0.001864	32.25

	2	78	0.00842	0.00825	0.00263	0.0185	0.00272	0.00183	32.30
	A	16	0.0084	0.00872	0.00321	0.0176	0.00339	0.00229	40.35
	I	62	0.00843	0.00815	0.00263	0.0185	0.00252	0.0017	29.89
	3	51	0.00889	0.00883	0.00228	0.0247	0.0035	0.00236	39.37
	A	19	0.00867	0.00904	0.00228	0.012	0.00207	0.0014	23.87
	I	32	0.00903	0.00840	0.00489	0.0247	0.00411	0.00277	45.52
	4	26	0.00786	0.00821	0.00302	0.0128	0.00267	0.0018	33.96
	A	13	0.00804	0.00822	0.00307	0.01225	0.00215	0.00145	26.74
	I	13	0.00768	0.00661	0.00302	0.0128	0.00311	0.00209	40.44
Popliteal lymph nodes.	II	294	0.25612	0.245	0.090	0.570	0.0769	0.05187	30.02
Actual.	1	137	0.26434	0.250	0.100	0.570	0.0751	0.05065	28.41
	2	80	0.24731	0.2375	0.100	0.465	0.0781	0.0527	31.58
	A	16	0.26218	0.250	0.135	0.440	0.0802	0.05409	30.58
	I	64	0.24359	0.235	0.100	0.465	0.0772	0.05207	31.69
	3	51	0.23974	0.235	0.090	0.450	0.0737	0.0497	30.74
	A	19	0.24063	0.237	0.090	0.420	0.0844	0.05693	35.07
	I	32	0.23921	0.2225	0.135	0.450	0.0667	0.04499	27.88
	4	26	0.2713	0.2725	0.145	0.450	0.0793	0.0535	29.22
	A	13	0.26946	0.270	0.155	0.450	0.0879	0.0593	32.62
	I	13	0.27308	0.290	0.145	0.400	0.0697	0.04701	25.52
Relative.	II	294	0.1425	0.1388	0.050	0.3335	0.04415	0.02978	30.98
	1	137	0.14653	0.139	0.050	0.3335	0.04511	0.03043	30.78
	2	80	0.13254	0.136	0.0563	0.2405	0.0382	0.02577	28.80
	A	16	0.13523	0.13835	0.0782	0.1965	0.0348	0.02347	25.70
	I	64	0.13186	0.13295	0.0563	0.2405	0.0396	0.0267	30.03
	3	51	0.13437	0.1375	0.0524	0.275	0.0431	0.02908	32.06
	A	19	0.13105	0.1415	0.0524	0.1965	0.0414	0.02792	31.57
	I	32	0.13635	0.137	0.0718	0.275	0.0441	0.02975	32.33
	4	26	0.1522	0.15325	0.0592	0.2645	0.0505	0.03406	33.18
	A	13	0.16302	0.1442	0.082	0.2645	0.0552	0.0372	33.86
	I	13	0.14136	0.153	0.0592	0.2035	0.0429	0.02894	30.34

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Axillary lymph nodes.	II	292	0.1632	0.157	0.070	0.485	0.0562	0.0379	34.43
Actual.	1	137	0.16767	0.160	0.070	0.485	0.06198	0.04181	36.96
	2	80	0.16257	0.160	0.075	0.340	0.0493	0.03325	30.31
	A	16	0.16281	0.165	0.085	0.235	0.0329	0.0202	22.19
	I	64	0.16251	0.1575	0.075	0.340	0.0526	0.03548	32.36
	3	51	0.15131	0.140	0.075	0.300	0.0476	0.0321	31.46
	A	19	0.14878	0.135	0.080	0.240	0.0461	0.0311	30.98
	I	32	0.15281	0.155	0.075	0.300	0.0483	0.0326	31.60
	4	24	0.16575	0.1675	0.070	0.310	0.0442	0.0298	26.65
	A	12	0.16733	0.1485	0.090	0.310	0.0625	0.0422	37.35
	I	12	0.16416	0.1800	0.070	0.210	0.0427	0.0288	26.01
Relative.	II	292	0.09005	0.0856	0.0341	0.240	0.0317	0.02138	35.20
	1	137	0.09257	0.0873	0.0359	0.240	0.03378	0.02278	36.49
	2	80	0.08813	0.0853	0.0341	0.1895	0.0277	0.0187	31.40
	A	16	0.08696	0.084	0.0341	0.1379	0.0225	0.01518	25.80
	I	64	0.08842	0.0853	0.0413	0.1895	0.0302	0.02037	34.15
	3	51	0.0836	0.0808	0.0439	0.1825	0.0255	0.0172	30.50
	A	19	0.08066	0.0806	0.0483	0.1165	0.02003	0.01351	24.83
	I	32	0.08535	0.0835	0.0439	0.1825	0.0282	0.01902	33.04
	4	24	0.09532	0.09175	0.0497	0.159	0.0316	0.0213	33.15
	A	12	0.10213	0.10045	0.0497	0.159	0.038	0.02563	37.20
	I	12	0.08851	0.08795	0.0516	0.133	0.0252	0.01699	28.47
Deep cervical lymph nodes.	II	289	0.1564	0.130	0.050	0.660	0.0787	0.05308	50.31
Actual.	1	136	0.1517	0.130	0.050	0.460	0.06502	0.04386	42.86

	2	78	0.16098	0.1375	0.070	0.660	0.09360	0.06313	58.14
	A	14	0.22785	0.185	0.125	0.560	0.11318	0.07634	49.67
	I	64	0.14635	0.1275	0.070	0.660	0.05454	0.03679	37.26
	3	49	0.16283	0.140	0.075	0.360	0.0648	0.04371	39.80
	A	17	0.17494	0.170	0.100	0.255	0.0524	0.03534	29.95
	I	32	0.1564	0.130	0.075	0.360	0.07001	0.04722	44.76
	4	26	0.1554	0.1275	0.060	0.560	0.1113	0.07507	71.62
	A	13	0.190	0.135	0.090	0.560	0.1468	0.09902	77.26
	I	13	0.1207	0.125	0.060	0.175	0.0276	0.0186	22.86
Relative.	II	289	0.0858	0.0738	0.0307	0.295	0.0414	0.0279	48.25
	1	136	0.08311	0.743	0.0319	0.221	0.03202	0.0216	38.52
	2	78	0.08566	0.0738	0.03398	0.295	0.04504	0.03038	52.57
	A	14	0.1163	0.0931	0.0656	0.252	0.05402	0.03643	46.44
	I	64	0.07896	0.07075	0.03398	0.295	0.0398	0.02685	50.40
	3	49	0.09084	0.0767	0.0456	0.227	0.0379	0.02556	41.72
	A	17	0.0959	0.097	0.0456	0.165	0.0336	0.02266	35.03
	I	32	0.08815	0.0718	0.0456	0.227	0.0401	0.02705	45.46
	4	26	0.0906	0.0704	0.0307	0.3361	0.0693	0.04674	76.49
	A	13	0.11533	0.076	0.0574	0.3361	0.0901	0.06077	78.12
	I	13	0.06589	0.0652	0.0307	0.0992	0.0162	0.01093	24.58
Mesenteric lymph nodes.	II	293	3.460	3.340	1.07	11.47	1.133	0.764	32.75
Actual.	1	136	3.515	3.335	1.07	11.47	1.260	0.850	35.84
	2	80	3.456	3.375	2.00	6.75	0.953	0.643	27.57
	A	16	3.150	3.170	2.20	4.27	0.533	0.360	16.92
	I	64	3.532	3.425	2.00	6.75	1.020	0.688	28.87
	3	51	3.450	3.350	1.20	6.20	1.060	0.710	30.70
	A	19	3.250	3.000	1.20	6.17	1.160	0.780	35.60
	I	32	3.560	3.500	2.00	6.20	0.987	0.666	27.72
	4	26	3.195	3.080	1.58	5.60	0.863	0.582	27.01
	A	13	3.215	3.100	2.27	4.35	0.601	0.405	18.69
	I	13	3.176	2.840	1.58	5.60	1.068	0.720	33.62

TABLE I—*Concluded.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Mesenteric lymph nodes. Relative.	II	293	1.915	1.830	0.67	6.91	0.642	0.433	33.52
	1	136	1.951	1.870	0.67	6.91	0.715	0.482	37.33
	2	80	1.856	1.815	0.902	3.65	0.589	0.397	31.73
	A	16	1.654	1.618	1.105	2.38	0.306	0.206	18.50
	I	64	1.907	1.833	0.902	3.65	0.628	0.424	32.93
	3	51	1.911	1.855	0.806	3.44	0.547	0.369	28.62
	A	19	1.758	1.635	0.806	3.09	0.527	0.355	29.97
	I	32	2.002	2.018	1.155	3.44	0.541	0.365	27.02
	4	26	1.834	1.653	0.936	2.88	0.508	0.343	27.69
	A	13	1.953	1.830	1.30	2.77	0.357	0.241	18.27
	I	13	1.714	1.64	0.936	2.88	0.532	0.359	31.03

The method employed in conducting the investigation and the classification of animals with reference to the extent of the lesions found at autopsy are described in detail elsewhere (1, 3). The same classification of material was followed in the separation of animals with active from those with inactive lesions. In this case, a primary division of animals was made according to the extent of the lesions found at autopsy and each of these groups was further divided on the basis of the activity of the lesions present. For this purpose, all animals presenting lesions that were healed or in an advanced stage of healing or resolution were classed as inactive, while those with any lesions which appeared to be progressing or showed no indication of healing were classed as active. We thus have one group of animals that showed no gross lesions at autopsy and three other groups with lesions that were slight, moderate, or marked; these three groups are subdivided into those with active and those with inactive lesions.

For convenience, the results of the statistical analysis of data are summarized in Table I which contains values for actual weights and for weights per kilo of net body weight (relative weight) as defined in a previous paper (3). Each set of figures gives the values obtained for the entire group of animals (295) and corresponding values for those with no lesions and those with active and with inactive lesions that were slight, moderate, or marked, in the order mentioned. These subgroups are designated by the arabic numerals 1, 2, 3, and 4; and the active and inactive divisions of each by the letters *A* and *I* respectively. The figures given in brackets for the thyroid in subgroup 2I, actual and relative, are values obtained by the omission of the weight of one large goiter. In Text-figs. 1 to 3, mean organ weights of the four subgroups of Series II are compared with corresponding values for the subgroups of Series I on the basis of the percentage deviation of the mean weight of a given organ from the mean weight for that organ for Series I or II as the case may be. Graphs of the coefficients of variation are given in Text-figs. 4 to 6.

Mean organ weights and coefficients of variation for animals with active and with inactive lesions are compared graphically in Text-figs. 7 to 15. In this case, the values are arranged in the form of a progressive series, reading from left to right. The first half of each text-figure gives the results for animals with active lesions arranged in the order of increasing abnormality, while the second half gives corresponding values for animals with inactive lesions arranged in the order of decreasing abnormality. The first and last values are those for strictly normal animals (no lesions found at autopsy).

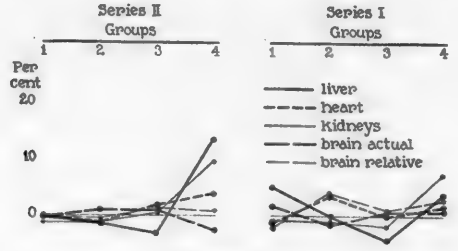
RESULTS.

The results are summarized in Table I and Text-figs. 1 to 15.

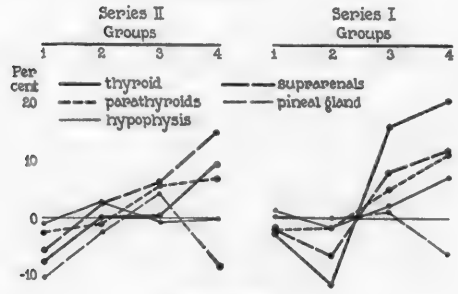
DISCUSSION AND CONCLUSIONS.

The prevalence of gross lesions among the animals of the present series (Series II) was somewhat greater than in the first (Series I)

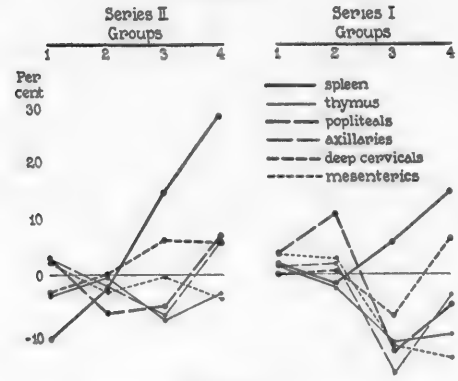
due to an increase in the number of animals with slight or moderate lesions; marked lesions were less frequent. The comparative per-



TEXT-FIG. 1.

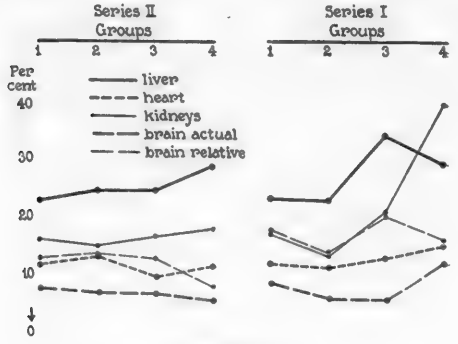


TEXT-FIG. 2.

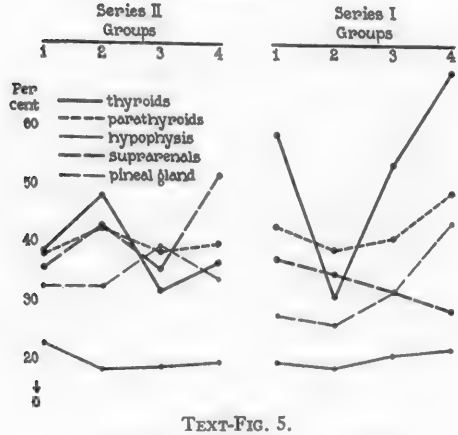


TEXT-FIG. 3.

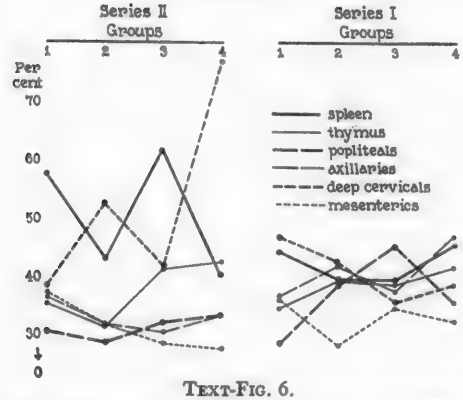
TEXT-FIGS. 1 to 3. Percentage deviation of mean organ weight from the mean weight of the series.



TEXT-FIG. 4.

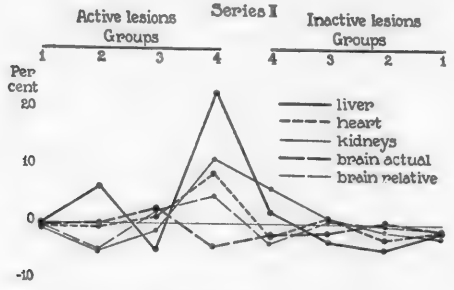


TEXT-FIG. 5.

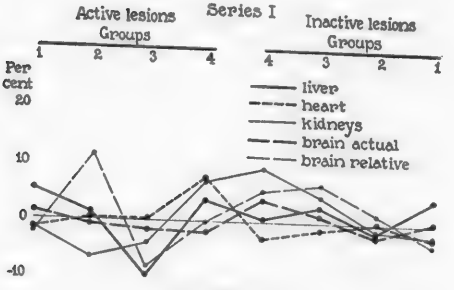


TEXT-FIG. 6.

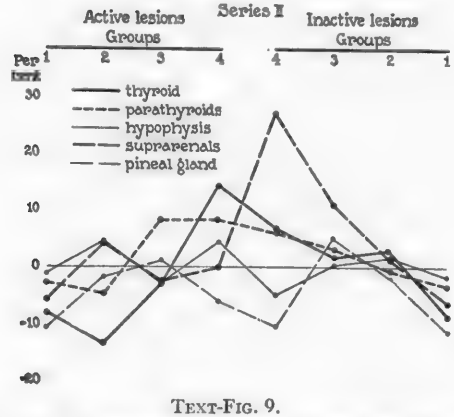
TEXT-FIGS. 4 to 6. Coefficients of variation for organ weight.



TEXT-FIG. 7.

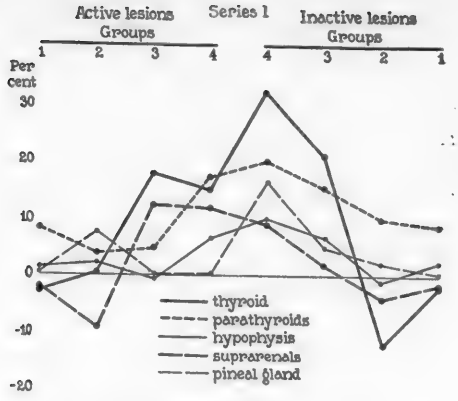


TEXT-FIG. 8.

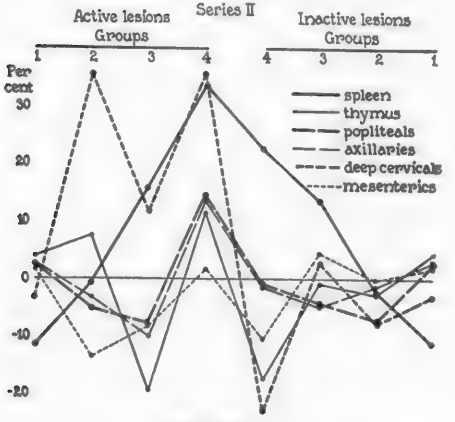


TEXT-FIG. 9.

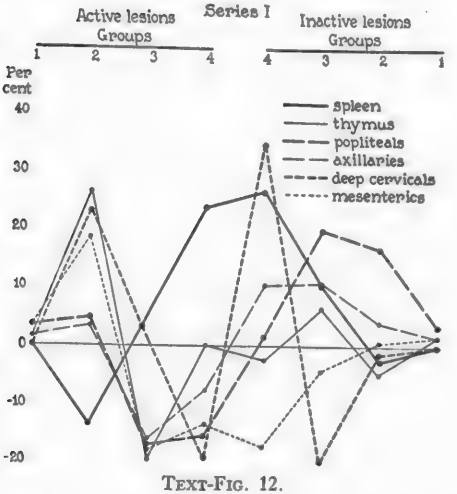
TEXT-FIGS. 7 to 12. Percentage deviation of mean organ weights from the mean for all animals of the series.



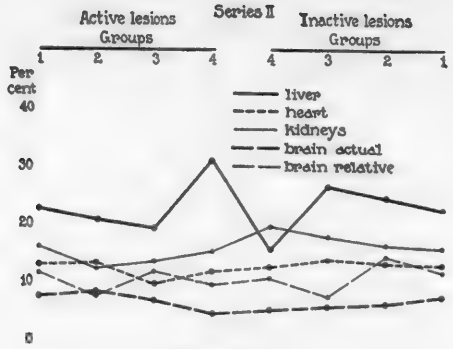
TEXT-FIG. 10.



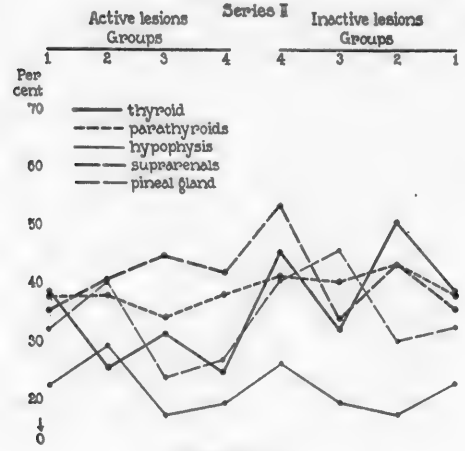
TEXT-FIG. 11.



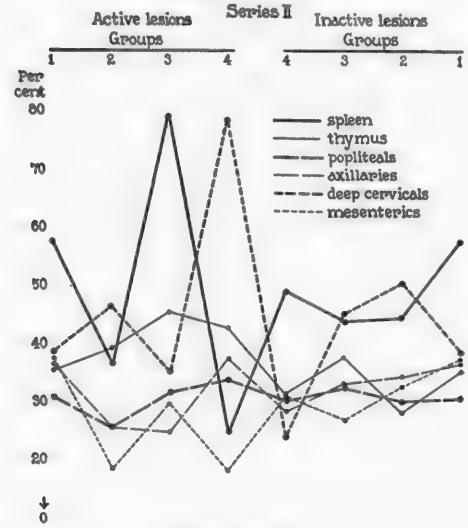
TEXT-FIG. 12.



TEXT-FIG. 13.



TEXT-FIG. 14.



TEXT-FIG. 15.

TEXT-FIGS. 13 to 15. Coefficients of variation for organ weight.

centage distribution of animals among the four subgroups and the combined incidence (645 rabbits) were as follows:

Group.	Series I.	Series II.	Total.
	per cent	*per cent*	*per cent*
1	59.1	46.8	53.49
2	20.3	27.1	23.41
3	10.3	17.3	13.49
4	10.3	8.8	9.61

The incidence of gross lesions in the entire group of 645 apparently healthy stock rabbits was, therefore, approximately 50 per cent (46.51 per cent); the extent of the lesions varied within comparatively narrow limits and only a small proportion of the animals (9.61 per cent), or about 1 in 10, showed extensive lesions, either active or healed.

The apparent effects of the lesions, or disease processes, on organ weights, as measured by mean weights and the tendency to variation, were of the same general order as in the first series of animals studied, and while the values obtained were not exactly the same in the two cases the agreement is sufficiently close to indicate that the results are significant. By reference to Text-figs. 1 to 3, it will be seen that the direction of the effect of marked lesions on mean organ weights and coefficients of variation as compared with normal animals (Groups 1 and 4) is usually the same. If the weight is increased in one case it is usually increased in the other or if it is diminished in one case it is diminished in the other, but the magnitude of the change differs and there is greater variation in the apparent effects of slight or moderate lesions. The agreement or disagreement between the two sets of animals (Series I and II) may be brought out by a tabulation of the distribution of maximum and minimum mean weights (per kilo of net body weight) and of coefficients of variation according to groups (Table II).

Table II shows that, with very few exceptions, the presence of obscure lesions was associated with an increase in the mean weight of organs per kilo of body weight; the two striking exceptions are the thymus and the mesenteric lymph nodes. There is less certainty concerning the relation of obscure lesions to the uniformity of organ

weights (coefficients of variation). The graphs (Text-figs. 4 to 6) indicate a closer agreement than the tabulated results, which again emphasizes the fact that the nature of the effect is more constant than the magnitude.

With reference to the apparent discrepancies between the results obtained for Series I and II, attention may be called to the fact that there were certain important differences in the material which un-

TABLE II.

Distribution of Maximum and Minimum Mean Weights and Coefficients of Variation According to Series and Groups.

Organ.	Mean weight.				Coefficient.			
	Maximum.		Minimum.		Maximum.		Minimum.	
	I	II	I	II	I	II	I	II
Thyroid	4	4	2	1	4	2	2	3
Hypophysis	4	2	2	1	4	1	2	2
Suprarenals	4	4	2	1	4	2	3	3
Parathyroids	4	4	1	1	4	2	2	1
Pineal gland	3	3	4	1	3	4	2	2
Popliteal lymph nodes	2	4	3	2	3	4	1	2
Axillary " "	2	4	3	3	4	1	1	3
Mesenteric " "	1	1	4	4	1	1	2	4
Thymus	1	1	3	3	4	4	1	2
Heart	2	4	1	2	4	2	2	3
Brain	2	3	1	2	3	2	2	4
Liver	1	4	3	3	3	4	2	1
Kidneys	4	4	3	1,2	4	4	2	2
Spleen	4	4	2	1	4	3	3	4
Deep cervical lymph nodes	4	3	3	1	1	4	3	1

doubtedly affected the results to some extent. In the first place, the nature and extent of the lesions presented and the distribution of different types of disease among the various subgroups were not the same in the two cases. Moreover, it will be noted that the normal values for Series II (Group 1) are lower on the whole than the corresponding values for Series I, which indicates the existence of a different state of organ balance due to the action of factors other than disease.

Differences of this kind may have some influence on the apparent effect of slight, moderate, or marked lesions on organ weight. For

example, it will be seen by reference to Text-fig. 2 that in the case of animals of Series I with slight lesions (Group 2), the weights of certain organs diminished to a point comparable to that of the animals in Group 1 of Series II before the upward movement took place. It will also be seen that the decided reduction in the weights of the thyroid and suprarenals of Series I (Text-fig. 2) was offset by a corresponding increase in the weights of lymph nodes (Text-fig. 3) and that the reduction and subsequent increase in the weights of the lymph nodes in animals with more extensive lesions was associated with a greater and corresponding increase in the weights of thyroids and suprarenals.

Such differences as these suggest that the apparent effect of a given stimulus may vary, depending upon the state of the organ (or organism) during the time the stimulus is operative or upon the effect produced by other stimuli. When we take into account even the known differences between the corresponding groups of animals of the two series, the extent of the agreement is surprising, especially so in view of the comparatively small absolute difference in the magnitude of the values with which we are dealing. It is obvious, however, that, as even the direction of the change is subject to some variation, less significance can be attached to the magnitude of the effect produced.

A comparison of results on the basis of the activity of the lesions furnishes further confirmation of the occurrence of alterations in organ weight in response to the action of disease processes which produce no clinical manifestations of disease. In addition, it appears that there are some differences between the effects of lesions that are active and those that are inactive as defined above. In Text-figs. 7 to 15, the results have been arranged so as to convey the idea of a progressive increase in the extent of the disease (reading from left to right) with a change from an active to an inactive state and a progressive reduction in the extent of the lesions with a return to normal. This is, of course, only a schematic representation with a wide gap at the point of transition from active to inactive lesions.

Here again the general direction of the curves from Group 1 to Group 4 and from 4 to 1 is of more importance than the magnitude of the difference between any two points. The curves for mean weight, in general, bear a relation to the extent and state of the lesions as

plotted. The results show essentially the same departure from and return to normal as was found in the case of rabbits presenting clinical manifestations of disease (4).

The curves for the coefficients of variation are more irregular and are difficult to interpret. If we disregard point to point fluctuations, however, and consider only the general direction of the change from normal to the most marked condition of active disease and from the most marked inactive lesions back to normal (Groups 1 to 4 and 4 to 1) it will be seen that there are distinct differences between the variability of weight for the two major groups of animals and that, in general, the direction of the curves bears a relation to the curves for mean weight. As an example of this condition, reference to Textfigs. 11 and 15 will show that the weight of the spleen increases progressively in animals with active lesions and decreases in the same manner in animals with inactive lesions; but the coefficients of variations are extremely irregular owing chiefly to the value given by the animals of Group 3 (active). Still, the general direction of the curve is downward from Group 1 to Group 4 and is upward from this point to Group 1 (reading from left to right), or is just the reverse of the weight curve. This indicates that the increase in mean weight was accompanied by an increase in the uniformity of weights for individual organs (except in Group 3) and that as the weight of the spleen diminished or returned to normal there was also a reduction in the uniformity of weights. Other organs show analogous relationships between the two sets of curves. Moreover, it will be seen that in some cases the curves for coefficients of active and of inactive lesions agree with those for the coefficients of Series I or II and that some of the discrepancies between the curves of these two series may be accounted for by the activity of the lesions of different groups of animals.

The results of this study of organ weights and obscure disease conditions in normal rabbits may be summarized briefly by saying that it furnishes conclusive evidence of a close relation between physical constitution, as represented by organ weight, and susceptibility to disease, as expressed by the extent and the activity of the lesions found post mortem. The evidence available indicates that disease, even in its mildest form, is capable of affecting the weights

of organs that are not directly involved by the disease process and that the effect produced bears a relation to both the extent and the activity of the lesions present. There is some evidence that the converse may be true also; namely, that the occurrence and subsequent course of disease are influenced in some measure by the state of organ balance and the ability of the animal organism to adjust itself to meet the demands of disease-producing agencies.

SUMMARY.

A study of organ weights was made on a group of 295 normal rabbits for the purpose of determining whether any relation could be detected between the weights of organs and the extent and activity of the lesions found post mortem.

The results obtained seemed to indicate that disease, even in its mildest form, is capable of affecting the weights of organs that are not directly involved by the disease process and that the effect produced bears a relation to both the extent and the activity of the lesions present. There was also some evidence that the converse might be true; namely, that the occurrence and subsequent course of disease may be influenced in some measure by the state of organ balance and the ability of the animal organism to adjust itself to meet the demands of disease-producing agencies.

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A BIOLOGICAL CLASSIFICATION OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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In a previous study (1) of the classification of the *encapsulatus* group of organisms certain technical difficulties were encountered which made a serological differentiation of these bacilli confusing and impracticable. The data presented at that time suggested the existence of two types, but these were ill defined and comprised only a small number of all the strains studied. The difficulties attending the immunological classification were ascribed to the failure to obtain potent sera and to the interference of the capsule of the organisms with agglutination. Numerous other investigators had made unsuccessful attempts at a classification, also, and for the most part they have attributed their failures to one or both of these causes.

A review of the literature reveals how contradictory and confusing the data are on the subject. Kraus (2) and Landsteiner (3) were the first to demonstrate agglutination in the *encapsulatus* group. Both investigators procured sera which agglutinated only in low dilutions. Sicard (4), Defalle (5), Clairmont (6), and Porges (7) obtained antisera inconstantly. Immune sera were procured by Klemperer and Scheier (8) and Bertarelli (9) but the sera were not specific and agglutinated closely related species. Specific sera were obtained by Friel (10), Coulter (11), Small and Julianelle (1), and Ball (12).

Howard (13) protected guinea pigs against Friedländer infection by active immunization, but the serum of these animals conferred no passive protection on normal animals. Landsteiner, Sicard, and Clairmont also were unable to demonstrate protective antibodies in the sera of immunized animals. Perkins (14) found that guinea pigs surviving a spontaneous Friedländer epidemic acquired immunity against the organism isolated during the epidemic. In a later paper (15) he states that "animal inoculation, immunization and agglutination show results which are far too variable to admit of them as means for classification."

Limiting their work to the differentiation of the Friedländer and rhinoscleroma organisms, Erben (16), Ballner and Reibmayr (17), and Galli-Valerio (18, 19)

could determine no serological differences between these organisms. Goldzieher and Neuber (20), on the other hand, obtained fixation reactions with *B. rhinoscleromatis*, using serum from patients suffering from rhinoscleroma and from rabbits immunized to the rhinoscleroma bacillus, but they were not successful when Friedländer's bacillus was used as antigen with the same sera. Babes (21) produced in rabbits an agglutinating serum of low titer against a strain of *B. rhinoscleromatis*. Sdrawosmysloff (22) found no complement-fixing antibodies in the serum of a rhinoscleroma patient.

Schmidt (23), using the serum of a patient convalescent from Friedländer infection of the lung, and the organism isolated from the sputum and from the lung by direct puncture, observed an "amorphous" agglutination. In this instance, no immune properties were demonstrable until after the 1st month of illness, when agglutination, bacteriolysis, and thread reaction were observed. Wolf (24) isolated a strain of Friedländer's bacillus from the urine of a patient whose serum agglutinated equally well the homologous strains and several stock laboratory ones in dilutions of 1:2000 and 1:5000.

Porges attributed the difficulty of immunization with *encapsulatus* strains to the presence of capsules which he thought interfered also with agglutination. Consequently he devised a method by which the capsular material was hydrolyzed by heating suspensions of the bacteria in weak acid. With von Eisler (25) he showed that after destruction of the capsule immune sera could be obtained and that agglutination was facilitated in this way. Streit (26) and Beham (27) took exception to the method because suspensions so treated were apt to clump spontaneously. They effected decapsulation by cultural methods, and obtained agglutination regularly.

Fitzgerald (28) found that immunization of rabbits with *B. rhinoscleromatis* yielded a potent serum which agglutinated four other strains of the organism equally well. These strains were agglutinated only when capsule-free. Small and Julianelle observed that capsule-bearing strains agglutinated only in high concentrations of immune sera, and the flocculation resulting under these conditions gave a compact disk at the bottom of the tube with a clear supernatant fluid. Capsule-free strains on the other hand agglutinated in serum dilutions as high as 1:2500 and gave a finely granular precipitate which was easily broken up. The encapsulated strains, moreover, adsorbed the specific agglutinins from the immune sera, while capsule-free strains did not.

It becomes evident from this review of the literature that great confusion exists as to the immunological identity of members of the *encapsulatus* group. The impression is that agglutination not only is difficult, but unreliable and not sharply differential. It seemed not unlikely, however, that the application to this group of the principles governing the immunological relationships of pneumococci and their cell constituents might afford a basis for determining similar relations

among the encapsulated bacilli. This newer concept of the bacterial cell (29–34) involves two separable and distinct constituents—the one a polysaccharide, the soluble specific substance, which endows the cell with type specificity; the other a protein substance, which, regardless of type derivation, exhibits immunologically only the common and undifferentiated characteristics of the species. The dissociation of the polysaccharide from the cell deprives the organism of its type-specific antigenic power. In general, antipneumococcus serum contains both the type-specific and the species-specific antibody; the occurrence of the latter depending upon the extent of the dissociation of the antigenic complex, which may take place both *in vitro* and *in vivo*. The presence of the protein antibody in appreciable amounts may therefore mask and sometimes obliterate the type specificity of an organism. Moreover, under unfavorable conditions, these organisms are known to lose the function of elaborating the soluble specific substance—a condition resulting in loss of type specificity, capsule formation, and accompanied by loss of virulence. These degraded strains lose the property of stimulating the type-specific antibody and as antigen provoke only the common protein response (also Stryker (35), Reimann (36), Amoss (37)). In other words, type specificity is demonstrable only when the antisera are dominantly type-specific and the strains to be agglutinated must be not degraded but encapsulated. Stripping an organism of its capsule therefore will minimize the specific serologic reactivity of the organism. It was with this conception, then, that the problem of the classification of the Friedländer bacilli was subjected to further analysis.

EXPERIMENTAL.

Strains of Microorganisms.—The organisms used in this investigation were all members of the *encapsulatus* group as determined by their individual, cultural, staining, and biochemical characteristics. They had been isolated at different times and places within the past 6 years. Strains E and K were isolated from guinea pigs with pneumonia; Strains H1 and H2 were derived from infections in horses; and the remaining strains, from human sources, the majority of them having been associated with lobar pneumonia. Two strains of *Encapsulatus granulomatis*, and two strains of *B. aerogenes* were included in the agglutination tests as a control of specificity. All the strains had in common the production of mucoid, gummy growth, though there were variations in this respect by the individual strains.

Immunization.—Normal rabbits were bled before immunization was begun and the normal sera were pooled and used for control in the subsequent tests. The animals were immunized by intravenous injections of suspensions of the heat-killed encapsulated organisms. Small doses were injected in the beginning and the amount increased during the course of the injections. Immunization was effected by injecting the organisms on 3 successive days, followed by a rest period of 4 days. Before each course of injections, the titer of the serum of each rabbit was determined; and when the serum showed no further increase in titer, immunization was discontinued. The rabbits were bled 9 or 10 days after the last injection. By this method, an efficient agglutinating serum was obtained in from 4 to 5 weeks.

Agglutination.—Agglutinations were conducted with saline suspensions of living bacilli kept without preservative. The various suspensions were diluted to an equal turbidity before being used for the test. Incubation was carried out for 2 hours at 37°C. in the water bath, and final readings were made after the tubes had been in the ice box overnight. In the more concentrated sera, positive results were usually observable within a few minutes. In agglutinin adsorption tests, heat-killed organisms were used.

Protection.—The protective power of the sera was determined by injecting white mice intraperitoneally with a mixture of 0.2 cc. of the serum and varying dilutions of a broth culture of the organism, both being brought to a volume of 0.5 cc. with sterile broth. The culture used was grown from 4 to 6 hours. The mice were kept under observation for 6 days and those surviving for this period were considered effectively protected.

Precipitin Tests.—In the precipitin tests 0.2 cc. of immune serum diluted to a volume of 0.5 cc. with salt solution was added to an equal volume of increasing dilutions of the soluble specific substance of Friedländer's bacillus. The mixtures were incubated for 2 hours at 37°C. in the water bath. Final readings were made on the following morning after the mixtures had been overnight in the ice box.

Thread Reaction.—In performing these tests, dilutions of the various sera were made in sterile broth. The serum broth tubes were then inoculated with 0.1 cc. of a 4 hour culture of the organisms to be studied and incubation was carried on overnight, for a period of 16 to 18 hours.

Agglutination.

Rabbits were immunized as described to seven of the strains studied. Use was also made of Type II antipneumococcus serum, in view of the recent work of Avery, Heidelberger, and Goebel (38).

The type-specific agglutinations are characteristic and result in a voluminous, compact disk at the bottom of the tube with a clear supernatant fluid. In the lesser dilutions the flocculation occurs immediately and becomes apparent even before incubation.

TABLE I.

Cross-Agglutination Reactions with Strains of Friedländer's Bacillus.

Strain of organism.	Immune sera—final dilution 1:5.								Normal Serum.
	F5	Sc	F2	F6	Pn. II	F10	F11	F12	
F3	++++	++++	−	−	−	−	−	−	−
F5	++++	++++	−	−	−	−	−	−	−
F8	++++	++++	−	−	−	−	−	−	−
F9	++++	++++	−	−	−	−	−	−	−
F15	++++	++++	−	−	−	−	−	−	−
F18	++++	++++	−	−	−	−	−	−	−
F19	++++	++++	−	−	−	−	−	−	−
F20	++++	++++	−	−	−	−	−	−	−
F21	++++	++++	−	−	−	−	−	−	−
F23	++++	++++	−	−	−	−	−	−	−
F25	++++	++++	−	−	−	−	−	−	−
Bu	++++	++++	−	−	−	−	−	−	−
Sc	++++	++++	−	−	−	−	−	−	−
Sm	++++	++++	−	−	−	−	−	−	−
St	++++	++++	−	−	−	−	−	−	−
F2	−	−	++++	++++	++++	−	−	−	−
F6	−	−	++++	++++	++++	−	−	−	−
E	−	−	++++	++++	++++	−	−	−	−
H1	−	−	++++	++++	++++	−	−	−	−
H2	−	−	++++	++++	++++	−	−	−	−
K	−	−	++++	++++	++++	−	−	−	−
F10	−	−	−	−	−	++++	−	−	−
M	−	−	−	−	−	++++	−	−	−
R	−	−	−	−	−	++++	−	−	−
F11	−	−	−	−	−	−	++++	−	−
F12	−	−	−	−	−	−	−	++++	−
F1	−	−	−	−	−	−	−	−	−
F13	−	−	−	−	−	−	−	−	−
F22	−	−	−	−	−	−	−	−	−
F24	−	−	−	−	−	−	−	−	−
G1	−	−	−	−	−	−	−	−	−
G2	−	−	−	−	−	−	−	−	−
A	−	−	−	−	−	−	−	−	−
W	−	−	−	−	−	−	−	−	−

++++ indicates complete, compact agglutination with clear supernatant; − indicates no agglutination.

Strains G1 and G2 are strains of granuloma bacillus, and A and W, *aerogenes*.

None of these strains were agglutinated by Type I or III antipneumococcus sera. The agglutination titer of these sera was as follows: F5, 1:40; Sc, 1:40; F2, 1:80; F6, 1:40; Pn. II, 1:40; F10, 1:40; F11, 1:160; F12, 1:80.

The cross-agglutination reactions are summarized in Table I. It becomes evident from the data that anti-Friedländer sera cause a highly specific agglutination which serves to define distinct type relations. Three types are revealed. Of the thirty strains included in

TABLE II.

Agglutinin Adsorption Reaction.
Result of Agglutination with Type A Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type A (F5).						
		Unadsorbed.	Adsorbed by strains of					
			Type A.					Type B.
			F5	F15	Sc	Sm	Bu	II
F3	A	++++	−	−	−	−	−	++++
F5	A	++++	−	−	−	−	−	++++
F8	A	++++	−	−	−	−	−	++++
F9	A	++++	−	−	−	−	−	++++
F15	A	++++	−	−	−	−	−	++++
F18	A	++++	−	−	−	−	−	++++
F19	A	++++	−	−	−	−	−	++++
F20	A	++++	−	−	−	−	−	++++
F21	A	++++	−	−	−	−	−	++++
F23	A	++++	−	−	−	−	−	++++
F25	A	++++	−	−	−	−	−	++++
Sc	A	++++	−	−	−	−	−	++++
Sm	A	++++	−	−	−	−	−	++++
St	A	++++	−	−	−	−	−	++++
Bu	A	++++	−	−	−	−	−	++++
E	B	−	−	−	−	−	−	−

The final dilution of serum in these tests was 1:5.

++++ indicates complete, compact agglutination; − indicates no agglutination.

the survey, twenty-four fall into one or another of these types. For the sake of convenience, these groups will be referred to as Type A, which is composed of fifteen strains; Type B, which is composed of six strains; Type C, which comprises three strains. The remaining six cultures, against four of which no sera were prepared, are placed in a

heterogeneous group, Group X. It is not unlikely that, with an extension of this study so as to include a greater number of strains,

TABLE III.

Agglutinin Adsorption Reaction.

Results of Agglutination with Type B Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type B (F2).				Strain.	Type.	Serum Type B (F6).			
		Unadsorbed.	Adsorbed by strains of					Unadsorbed.	Adsorbed by strains of		
			Type B.		Type A.				Type B.		Type A.
			F2	E	Sc				F6		Sc
F2	B	++++	−	−	++++	F2	B	++++	−	−	++++
F6	B	++++	−	−	++++	F6	B	++++	−	−	++++
E	B	++++	−	−	++++	E	B	++++	−	−	++++
K	B	++++	−	−	++++	K	B	++++	−	−	++++
H1	B	++++	−	−	++++	H1	B	++++	−	−	++++
H2	B	++++	−	−	++++	H2	B	++++	−	−	++++
Sc	A	−	−	−	−	Sc	A	−	−	−	−

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with Type C Serum after Adsorption with Strains of Homologous and Heterologous Types.

Strain.	Type.	Serum Type C (F10).				
		Unadsorbed.	Adsorbed by strains of			
			Type C.			Type A.
			F10	M	R	Sc
F10	C	++++	−	−	−	++++
M	C	++++	−	−	−	++++
R	C	++++	−	−	−	++++
Sc	A	−	−	−	−	−

additional types may be demonstrated among the heterogeneous strains of Group X.

The B type of the Friedländer bacillus is similar immunologically to Type II Pneumococcus. Avery, Heidelberger, and Goebel have already called attention to this relationship. It will be recalled that they showed that certain strains of Friedländer's bacillus, now designated as Type B, possess specific polysaccharides which, both in chemical and immunological properties, are so similar as to show cross-agglutination and protection, but no reciprocal adsorption of antibodies. It is seen that the six strains of this type react as well with Type II antipneumococcus serum as they do with their homologous sera.

TABLE V.

Protective Action of Anti-Friedländer Serum, Type A, against Strains of Homologous and Heterologous Types.

Amount of culture.	Immune serum Type A.	Strains of Friedländer bacillus.			
		Type A.			Type B.
		F5	F9	Sc	E
cc.	*cc.*				
10^{-3}	0.2	D. 23 hrs.	D. 48 hrs.	D. 24 hrs.	D. 16 hrs.
10^{-4}	0.2	S.	S.	S.	" 40 "
10^{-5}	0.2	"	"	"	" 40 "
10^{-6}	0.2	"	"	"	" 90 "
10^{-5}	0	D. 15 hrs.	D. 24 hrs.	D. 40 hrs.	D. 16 hrs.
10^{-6}	0	" 20 "	" 45 "	" 45 "	" 44 "
10^{-7}	0	" 40 "	" 46 "	" 46 "	" 40 "

S. indicates that the animal survived; D., that the animal died. The numerals represent the number of hours before death occurred.

Agglutinin Adsorption.

Further evidence of the specificity of the types just mentioned was obtained by agglutinin adsorption tests. The type sera were adsorbed with heat-killed suspensions of both homologous and heterologous type strains. When all the antibodies had been removed for the adsorbing strain, the sera were tested for the presence of agglutinins for the other strains of the homologous type.

The data summarized in Table II show that Serum F5 (Type A)

was adsorbed with five homologous strains, F5, Sc, F15, Sm, and Bu, and in each case the agglutinins were completely adsorbed. Adsorption with a heterologous strain, E, left untouched the agglutinins in this serum. When Serum F2 (Type B) was adsorbed with Strains F2 and E, homologous strains, agglutinins were removed from the serum not only for the adsorbing strains but for all the strains which were agglutinated by the unadsorbed serum. Strain Sc, a heterologous type, however, exerted no influence on the agglutination of the strains of the type serum. Similarly, adsorption of Serum F6 (also Type B) with

TABLE VI.

Protective Action of Anti-Friedländer Serum Type B, against Strains of Homologous and Heterologous Types.

Amount of culture.	Immune serum Type B.	Strains of Friedländer bacillus.			
		Type B.			Type A.
		F6	K	E	F9
cc.	*cc.*				
10^{-2}	0.2	D. 23 hrs.	—	—	—
10^{-3}	0.2	S.	D. 40 hrs.	D. 40 hrs.	D. 19 hrs.
10^{-4}	0.2	"	S.	S.	" 23 "
10^{-5}	0.2	"	"	"	" 92 "
10^{-6}	0.2	"	"	"	" 40 "
10^{-5}	0	D. 44 hrs.	D. 16 hrs.	D. 16 hrs.	D. 24 hrs.
10^{-6}	0	" 48 "	" 18 "	" 44 "	" 40 "
10^{-7}	0	S.	" 21 "	" 44 "	" 44 "

homologous strains removed all agglutinins for members of this group, whereas, adsorption by Sc, a heterologous strain, again had no effect on the agglutinin titer (Table III). Serum F10 (Type C) was no longer agglutinating for its three homologous strains after adsorption had taken place by Strains F10, M, and R. Strain Sc (Type A), however, adsorbed out none of the agglutinins (Table IV). The results obtained by the method of agglutinin adsorption constitute conclusive evidence that the types disclosed by agglutination with anti-Friedländer sera are real types.

Protection Tests.

That Friedländer bacilli are separable into at least three specific types, as demonstrated by the reactions of agglutination and agglutinin adsorption is a fact which gains further demonstration from animal protection tests. It is evident from the data presented in Tables V and VI that the protective action of the type immune sera reveals type specificity in the case of Friedländer bacilli just as sharply as does agglutination. Type A serum (Strain F5) protected mice against homologues of this type to an infective dose of 0.0001 cc. when the cultures themselves killed white mice regularly in a dilution of 0.0000001 cc. within 48 hours. On the other hand it offered no protection against an infection with an organism of a different type. Type B serum (F2) protected against infection by three strains of the homologous type to 0.0001 cc. when the cultures as such were fatal to a dilution of 0.0000001 cc.; yet this serum offered no protection against infection of a different type.

It was not possible to obtain data for the protective power of Type C sera. The three cultures of this type were avirulent for mice, and, despite numerous animal passages, the lethal dose could not be increased. The strains were all capsule-bearing and no "R" colonies could be demonstrated by plating directly from the peritoneal fluid of infected mice.

It is obvious from the results of the protection test that certain anti-Friedländer sera offer definite protection against infection of the organisms and that the protection is type-specific. The protective power of these sera was not as high as had been anticipated however. Avery, Heidelberger, and Goebel obtained a greater degree of protection against Type B infection; but there is no essential discordance between their results and ours, since our animals did not receive as intensive an immunization.

Thread Reaction.

Still further proof of the type specificity of the Friedländer organisms was obtained by means of the thread reaction. This reaction depends upon the character of the growth of an organism in homologous immune serum. Although the mechanism of the reaction may be

TABLE VII.

Thread Reaction.

Thread Formation during Growth of Friedländer's Bacillus in Presence of Immune Sera of Homologous and Heterologous Types.

Anti-Friedländer serum.		Strain Sc, Type A. Dilution of serum.						Strain E, Type B. Dilution of serum.						Strain M, Type C. Dilution of serum.						Broth control.
		1:5	1:10	1:50	1:100	1:500	1:1000	1:5	1:10	1:50	1:100	1:500	1:1000	1:5	1:10	1:50	1:100	1:500	1:1000	
Type A	F5	++++	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Type B	F2	−	−	−	−	−	−	++++	++++	++	±	−	−	−	−	−	−	−	−	−
	F6	−	−	−	−	−	−	++++	++	+	−	−	−	−	−	−	−	−	−	−
Type C	F10	−	−	−	−	−	−	−	−	−	−	−	−	++++	+++	+	−	−	−	−
Group X	F11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	F12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Normal serum.		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

++++ indicates growth was compact and completely sedimented with clear supernatant; +++ indicates marked, but not complete sedimentation; ++, clumping, more easily broken up; +, partial clumping; ±, slight clumping; −, no clumping.

the same as that involved in agglutination, it is usually more delicate than agglutination, and frequently positive results are obtained at a dilution of serum beyond the range of agglutination.

The findings are presented in Table VII. It is seen that a strain gives the thread reaction only when it is grown in immune serum of the homologous type. The reaction extends over the same range of dilutions as agglutination. It was expected that the thread reaction would be detectable in greater dilutions, but the growth of the organism is so profuse as probably to obscure the clumped growth that might occur at the higher dilutions.

Precipitin Reaction.

It has been stated earlier in this paper that a part of the difficulty in demonstrating types by serological reactions in the *encapsulatus* group has been explained on the basis that capsules inhibit agglutination and interfere with agglutinin formation in the body. The methods which were devised to strip the organism of its capsule, at the same time removed from the organism its specificity.

The first workers considered the capsule to be a nucleoprotein (39, 7, 28, 11). The chemical nature of the substance was studied by Rettger (40), who considered it a glycoprotein, possibly a pseudomucin. Toenniessen (41) isolated from a strain of Friedländer's bacillus a polysaccharide which yielded on hydrolysis an osazone, probably of galactose. Kramár (42) isolated a polysaccharide which on inversion yielded galactose. None of these investigators, however, recognized the relationship between the chemical nature and biological specificity of the capsular material.

Recently Mueller, Smith, and Litarczek (43) derived from a Friedländer bacillus a carbohydrate-containing material which reacted to a very high dilution of homologous immune serum. Heidelberger, Goebel, and Avery (44, 38) obtained a nitrogen-free polysaccharide from Strain E (Type B) which reacted specifically with immune serum of the homologous type in dilutions of 1:4 millions. Blake (45) was the first, and as far as we know the only one, to show the appearance of the soluble specific substance of Friedländer's bacillus in the blood and urine during an active infection. Although he could demonstrate no agglutinins in the serum of a patient, he found increasing amounts of the soluble specific substance of Friedländer's bacillus in both blood and urine. This is an interesting example of similarity in Friedländer and Pneumococcus pneumonia, since it is possible in both infections (46) to demonstrate the presence of the soluble specific substance of the causative organism in the blood and urine of patients suffering from severe infection.

Since the relation of capsular material and specificity has been recognized it seemed desirable to determine the action of immune sera of the different types on the isolated specific substance of Friedländer bacilli. Nitrogen-free polysaccharides of Strain Sc (Type A) and Strain E (Type B) were obtained[1] and cross-precipitin reactions were carried out in the usual manner. The amount of immune serum was kept constant at 0.2 cc. while the polysaccharide was used in increasing dilutions.

TABLE VIII.

Precipitation of the Soluble Specific Substance of Friedländer Bacilli by Immune Sera of Homologous and Heterologous Types.

Anti-Friedländer serum.		Type A soluble specific substance.								Type B soluble specific substance.								Salt control.
		20*	50	100	250	500	1000	2000	4000	20	50	100	250	500	1000	2000	4000	
Type A	F5	++	++	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−
	Sc	++	++	++	+	±	−	−	−	−	−	−	−	−	−	−	−	−
Type B	F2	−	−	−	−	−	−	−	−	+	++	++	++	+	+	±	−	−
	F6	−	−	−	−	−	−	−	−	+	++	++	+	+	±	−	−	−
	E	−	−	−	−	−	−	−	−	+++	+++	+++	++	+	±	−	−	−
Type C	F10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Group X	F11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	F12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Normal serum.		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

++++ indicates heavy compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity; ±, slight turbidity; −, no reaction:

* These figures represent dilutions in thousands.

The results of the precipitation tests are presented in Table VIII, and they show that the polysaccharide of each type reacts only with immune sera of the corresponding type and that precipitation occurs at a high dilution of the soluble specific substance. Further evidence to be reported in a subsequent publication will show that the organism

[1] The polysaccharides were obtained through the courtesy of Dr. Heidelberger and Dr. Goebel.

without its capsule is devoid of type specificity. It becomes obvious, therefore, that by removal of the capsule, specificity is lost.

DISCUSSION.

Although numerous attempts have been made by various investigators to classify the Friedländer group of bacilli on the basis of serological reactions, confusion rather than system has resulted. The newer conception of the immunological relationships of the capsular and somatic substances of the bacterial cell has furnished us, in part at least, with an explanation of the earlier failures. The principles involved are those brought out by the immunochemical studies on the type specificity of Pneumococcus, which have been carried on in this laboratory. The work has revealed a cellular mechanism in which two distinct and separable substances are concerned in the antigenic and serological behavior of the cell. One of these, the capsular or ectoplasmic layer of the cell, is the soluble specific substance which is now identified as a polysaccharide and is chemically different for each of the fixed types of Pneumococcus. The differential specificity of the bacterial types is dependent solely upon the presence of this substance.

That the carbohydrate fraction of the Friedländer bacillus is the substance which determines specificity among organisms of the *encapsulatus* group has been demonstrated in the present study, and in other papers from this laboratory. Removal of the capsule either by hydrolysis or by cultural methods results in a loss of specificity of the organism.

The second constituent of the Pneumococcus cell is the somatic protein substance which, regardless of type derivation, exhibits immunologically only the common and undifferentiated characteristics of the species. Immunization by a number of workers with a capsule-free Friedländer bacillus has yielded a serum which reacted with capsule-free strains of any type of these organisms.

Antigenically, the polysaccharide of the bacterial cell is inert. However, in the form in which it exists in the cell, this carbohydrate complex is the dominant and effective antigen yielding on immunization the corresponding type-specific antibody. It is now recognized that cell dissolution, whether occurring spontaneously in the body or arti-

ficially *in vitro*, is accompanied by more or less antigenic dissociation. The result of this process is reflected in the immune serum by the increasing amount of non-specific antibody with diminution or complete loss of type-specific response.

In previous studies it was observed that prolonged immunization induced a serum of high titer for the Friedländer organism, but that the serum agglutinated as well a number of organisms which were unable to remove agglutinins of the specific type by adsorption of the serum. Other workers also produced high titered sera at the expense of specificity.

The lack of recognition of two distinct antibodies in a single immune serum, each specifically reactive with only one of the cell constituents, and conversely the failure to distinguish the type-specific from the species-specific antigen of the cell, explain some of the difficulties encountered in the earlier efforts at classification of the *encapsulatus* group.

In a later paper the immunological relationships of these two constituents the protein and carbohydrate, and of encapsulated and capsule-free strains of the Friedländer bacillus, will be described.

SUMMARY.

A biological classification has been made of thirty strains of Friedländer's bacillus. This study reveals that there exist among these strains three sharply defined and specific types and one heterogeneous group. The three types are Type A, fifteen strains; Type B, six strains; Type C, three strains; and Group X, six strains. The agglutination, agglutinin adsorption, protection, thread, and precipitin reactions have been employed in the working out of this classification, and the types have been proved highly specific by means of each serological test.

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THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

III. The Production of Active and Passive Anaphylaxis in the Blocked Animal.

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INTRODUCTION.

With the increase of experimental work on the significance of the reticulo-endothelial tissue, especially that found in the liver and spleen, for the allergic reaction of the immune animal, the opinion seems to be more widely accepted that there is an intimate relationship between antibody production and the reticular elements of the organs mentioned.

Successful experiments to inhibit or suppress the formation of immune bodies (agglutinins, precipitins, hemolytic amboceptors) by blocking injections of India ink and other substances of colloidal dispersion, were reviewed thoroughly in a previous paper (1) in which the authors were able to show that no diphtheria antitoxin appeared in the blood during a period of 3 weeks in guinea pigs injected intravenously with massive doses of India ink before immunization with diphtheria toxin-antitoxin mixture. In a later paper (2), it was demonstrated that the complement titer of the serums of guinea pigs after intravenous injections of ink showed a marked transitory drop. Simultaneously, the regenerative power of the reticulo-endothelial cells of the liver and spleen of guinea pigs which had received one blocking injection of India ink, was measured by means of reduction tests. In these experiments it was found that, physiologically, the integrity of these tissues was restored in from 24 to 48 hours.

While the influence of intravenous injections of colloidal substances in the anaphylactic animal before reinjection of the antigen has recently been studied by a number of authors (3–8), it remains to be investigated whether massive doses of India ink, given before the sensitizing injection, will prevent or modify the sensitization of an animal. Aside from the earlier experiments of Mautner (9), more recently confirmed and amplified by Luzzato (10), which demonstrated the

importance of the spleen in sensitization, only short notes by Siegmund (11) and Neufeld (12) are found in the literature on the relation of the cells of the reticulo-endothelial system to the occurrence of anaphylaxis.[1] While Siegmund states that blocking of the reticulo-endothelial system led to the prevention of anaphylactic shock without, however, mentioning whether the blocking injections were given before or after sensitization, Neufeld reports that he has been unable to prevent sensitization in mice and guinea pigs by blocking and splenectomy carried out before the sensitizing injection.

In view of the meager and variable results obtained on the effect of blockade before sensitization in the occurrence of anaphylaxis, it seemed desirable to enter into a detailed study of the subject. Moreover, such work, if it were to include a quantitative titration of the anaphylactic power of the serum and a determination of the precipitin titer, would be of interest in throwing new light on the theory of the identity of precipitins and the anaphylactic antibody as advanced by Doerr (16). The study was therefore arranged to include the following points: (1) the effect upon the development of active anaphylaxis of India ink injected intravenously into guinea pigs prior to the sensitizing injection; (2) a determination of the precipitin titer and titration of the anaphylactic power of the serums of rabbits, injected intravenously with India ink prior to the sensitizing injections; (3) the effect upon the development of passive anaphylaxis in guinea pigs of intravenous injections of India ink given prior to the sensitizing injection. At the same time it was thought of interest not only to study the influence of massive blocking doses, but also of smaller doses of India ink, on the occurrence of these phenomena. Animals which had received massive doses of ink intravenously are frequently referred to later as "blocked," merely for the sake of brevity and not with the intention of making a definite statement as to the mode of action of the ink.

[1] Since this study was completed, Fujioka (13), Isaacs (14), and Simitch (15) have reported experimental work on the same subject. Isaacs has seen no shock-preventing action of trypan blue given either before or after sensitization in actively sensitized guinea pigs. Likewise Simitch observed that after blocking with such substances as iron oxide and India ink before sensitization, the occurrence of anaphylaxis was not interfered with. Fujioka, on the other hand, found that after experimental disturbance of functions of the reticulo-endothelial cells by the administration of lanolin emulsion, both active and passive anaphylaxis could be prevented.

TABLE I.

Series 1.

Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink.*			Sensitizing injections of normal horse serum No. 140 on June 15.†	Reinjections of normal horse serum No. 140 on July 6.*	Results.
	Date.	Dilution.	Dose.	Dose.	Dose.	
			cc.	*cc.*	*cc.*	
1				0.05	0.175	Moderate symptoms. Survived.
2				0.05	0.175	" " "
3				0.05	0.2	Died in 3 min.
4				0.05	0.2	" " 5 "
5				0.05	0.2	" " 3 "
6				0.05	0.2	" " 8 "
7	June 7	1:5	1.5	0.05	0.2	" " 3 "
	" 12	1:5	1.5			
8	" 7	1:5	1.5	0.05	0.2	Moderate symptoms. Died in 40 min.
	" 12	1:5	1.5			
9	" 7	1:5	1.5	0.05	0.2	Severe symptoms. Died in 2 hrs.
	" 12	1:5	1.5			
10	" 7	1:5	1.5	0.05	0.2	Slight symptoms. Died in 22 hrs.
	" 12	1:5	1.5			
11	" 7	1:5	1.5	0.05	0.2	Severe symptoms. Died in 20 min.
	" 12	1:5	1.5			
12	" 7	1:5	1.5	0.05	0.2	Moderate symptoms. Survived.
	" 12	1:5	1.5			
13	" 12	1:20	1	0.05	0.2	Died in 5 min.
14	" 12	1:20	1	0.05	0.2	" " 6 "
15	" 12	1:20	1	0.05	0.2	" " 3 "
16	" 12	1:20	1	0.05	0.2	" " 4 "
17	" 12	1:20	1	0.05	0.175	" " 4 "
18	" 12	1:20	1	0.05	0.175	" "10 "

The symptoms consisted of the classical syndrome, but varied in intensity. The autopsy findings were typical in each case of acute shock; lungs were maximally inflated; congestion of abdominal organs was present; heart's blood was found not clotted.

* All blocking injections and reinjections were intravenous.

† All sensitizing injections were subcutaneous.

EXPERIMENTAL WORK.

A. Experiments on the Effect of Blockade before Active Sensitization on the Occurrence of Anaphylactic Shock in Guinea Pigs.

First Series.—Six guinea pigs of from 230 to 280 gm. in weight were given two intravenous injections of 1.5 cc. India ink,[2] diluted with physiological salt solution 1:5; simultaneously, six guinea pigs received one intravenous injection of 1 cc. India ink, diluted 1:20. 3 days after the last ink injection, all twelve animals, together with six normal controls, were sensitized by one subcutaneous injection of 0.05 cc. of normal horse serum (1 cc. of a dilution 1:20). The intravenous reinjection was carried out 3 weeks later. Great care was taken to determine as accurately as possible the smallest dose of antigen which would kill a normal sensitized guinea pig with certainty, and this dose was later used for the reinjection of the ink-treated animals. This was necessary in order to facilitate the detection of very small changes in the degree of sensitization. The results obtained in this series are given in Table I.

It appears from this table that of the six blocked animals one survived, four died in a somewhat protracted shock, and only one died in 3 minutes with a dose (0.2 cc.) that, in from 3 to 4 minutes, produced typical shock in, and killed, the four non-blocked controls that received it. On the other hand, four out of six animals that had received the smaller dose of ink showed the same sensitiveness as the controls, when tested with the 0.2 cc. of normal horse serum, but the remaining two animals when injected with a dose sublethal for controls (0.175 cc.) died of acute shock in 4 and 10 minutes respectively. The results seem to indicate that under the conditions of the experiment massive doses of India ink injected intravenously before subcutaneous sensitization may effect a slight reduction in sensitiveness to the reinjection of the antigen 3 weeks later.

It was thought to be of interest to investigate whether the serum of the blocked guinea pigs which had exhibited a slightly decreased sensitiveness as compared with normal controls, contained enough anaphylactic antibody to make possible a passive transfer.

For this purpose, 4 cc. of blood were obtained by cardiac puncture from two blocked animals and from two controls 2 days before the reinjection in the ex-

[2] The technique of the intravenous injection was described in detail in the first paper. The India ink used was Higgins' insoluble India ink, which has been employed throughout these studies.

periment described above was carried out. 2 cc. of serum from each animal were injected intraperitoneally into each of four normal guinea pigs. These guinea pigs were reinjected intravenously 24 hours later with 1 cc. of normal horse serum. Simultaneously, one normal guinea pig was injected intravenously with the same amount to control the toxicity of the horse serum. Table II gives the results obtained.

This table shows that both blocked guinea pigs had developed, as had the controls, enough circulating anaphylactic antibody to make possible a passive transfer. The small number of animals in this case, however, makes the test only qualitative in nature and does not permit

TABLE II.

Passive Anaphylaxis in Normal Guinea Pigs with Serum from Sensitized Blocked and Normal Guinea Pigs.

Guinea pig No.	Sensitizing injection on July 5.*		Reinjections of normal horse serum No. 140 on July 6.†	Results.
	Serum from Guinea pig No.	Dose.	Dose.	
		cc.	cc.	
19	5	2	1	Died in 3 min.
20	3	2	1	" " 5 "
21	9	2	1	" " 3 "
22	10	2	1	" " 10 "
23			1	No symptoms.

Autopsy findings in each case were typical.

* All sensitizing injections were intraperitoneal.

† All reinjections were intravenous.

the quantitative comparison of the anaphylactic power of the serums of blocked animals with those of controls.

Second Series.—It was next thought to be of interest to investigate the effect on the occurrence of anaphylactic shock in guinea pigs of intravenous injections of India ink preceding sensitization, which was carried out intravenously. In view of the rapidity with which the reticulo-endothelial system recovers after blockade, it seemed also desirable to reduce the interval between blocking and sensitizing injections to the shortest possible time.

In this series, six guinea pigs, of from 250 to 280 gm. in weight, were given two intravenous injections of 1.5 cc. of a 1:5 dilution of ink at 6 day intervals and were

TABLE III.

Series 2.

Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution.*		Sensitizing injections of normal horse serum No. 140 on July 20.*	Reinjections of normal horse serum No. 140 on Aug. 10.*	Results.
	Date.	Dose.	Dose.	Dose.	
		cc.	*cc.*	*cc.*	
24			0.01	0.2	Slight symptoms. Survived.
25			0.01	0.5	Moderate symptoms. Survived.
26			0.01	0.5	" " "
27			0.01	0.75	Died in 3 min.
28			0.01	0.75	" " 10 "
29			0.01	0.75	" " 6 "
30	July 14 " 20	1.5 1.5	0.01	0.75	" " 3 "
31	" 14 " 20	1.5 1.5	0.01	0.75	" " 3 "
32	" 14 " 20	1.5 1.5	0.01	0.75	" " 4 "
33	" 14 " 20	1.5 1.5	0.01	0.75	" " 3 "
34	" 14 " 20	1.5 1.5	0.01	0.75	" " 3 "
35	" 14 " 20	1.5 1.5	0.01	0.5	Moderate symptoms. Survived.

Autopsy findings in each case were typical.

* All injections were intravenous.

sensitized 15 minutes after the last blocking injection by intravenous injection of 0.01 cc. of normal horse serum (1 cc. 1:100). At the same time, six normal controls were sensitized in the same manner. 3 weeks later the blocked animals were reinjected intravenously with the minimum dose of normal horse serum fatal to controls. The results obtained in this series are shown in Table III.

This table fails to disclose any reduction of sensitiveness in the blocked animals, as compared with the normal controls, to the reinjection of the antigen. With the dose of 0.75 cc., invariably fatal to the controls, all blocked animals also died typically, while the dose of 0.5 cc. was followed by the survival of two control animals and one blocked animal. There seems to be a fundamental difference between subcutaneously and intravenously sensitized guinea pigs as regards their susceptibility to anaphylactic shock following blocking injections of India ink given before sensitization.

Third Series.—Since the effect of blocking before sensitization apparently varies, it seemed desirable to run another series with a view to confirming the results obtained in the first series with subcutaneous sensitization. The extreme delicacy of the anaphylactic reaction as an indicator of antibody production, as manifested by the disproportion between the size of the sensitizing dose and the degree of change produced, subjects experiments on the inhibition of antibody production to a test of maximum severity. It was thought that by decreasing the antigenic dose to the smallest stimulus compatible with a regularly occurring sensitization and by testing for anaphylaxis before the condition had reached its maximum development, it should be possible to demonstrate better an inhibiting influence of the blockade on the formation of the anaphylactic antibody.

In this third series, six guinea pigs of from 250 to 280 gm. in weight, were given two intravenous injections of ink, as already described, and were sensitized 1 day after the last ink injection by a subcutaneous injection of 0.002 cc. of normal horse serum (1 cc. 1:500). Simultaneously, six control animals were sensitized with the same dose. Four blocked and four control animals were reinjected intravenously with the definitely fatal dose after an interval of 16 days, and the remaining four animals after 28 days. The results are shown in Table IV.

It appears from Table IV that in the first group, reinjected after 16 days had elapsed since the sensitization, two blocked animals survived and one died after the injection of a single lethal dose (0.1 cc.) which

TABLE IV.

Series 3.

Production of Active Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution.*		Sensitizing injections of normal horse serum on Oct. 27.†	Reinjections of normal horse serum.*		Results.
	Date.	Dose.	Dose.	Date.	Dose.	
		cc.	*cc.*		*cc.*	
36			0.002	Nov. 12	0.15	Died in 3 min.
37			0.002	" 12	0.1	" " 3 "
38			0.002	" 12	0.1	" " 4 "
39			0.002	" 12	0.05	Moderate symptoms. Survived.
40			0.002	" 24	0.1	Severe " Died in 20 hrs.
41			0.002	" 24	0.1	Died in 10 min.
42	Oct. 23 " 26	1.5 1.5	0.002	" 12	0.1	Moderate symptoms. Survived.
43	" 23 " 26	1.5 1.5	0.002	" 12	0.1	" " "
44	" 23 " 26	1.5 1.5	0.002	" 12	0.1	Died in 10 min.
45	" 23 " 26	1.5 1.5	0.002	" 12	0.15	" " 4 "
46	" 23 " 26	1.5 1.5	0.002	" 24	0.1	Severe symptoms. Survived.
47	" 23 " 26	1.5 1.5	0.002	" 24	0.1	Died in 8 min.

Autopsy findings were typical in each case, of acute shock.

* All blocking injections and reinjections were intravenous.

† All sensitizing injections were subcutaneous.

killed two controls in acute shock, while a slightly larger dose (0.15 cc.) produced fatal shock in one blocked animal.

In the second group, one out of two animals survived the injection of a single lethal dose (0.1 cc.) which killed two controls.

B. Determination of the Precipitin Titer and of the Anaphylactic Power of the Serum of Actively Sensitized, Blocked Rabbits.

In the work reported in the first part of this paper it was shown that guinea pigs blocked before active sensitization may exhibit occasionally a slightly lower sensitiveness to the reinjection of the antigen. By means of a rough qualitative test, it was demonstrable that the blocked animals, in spite of their inherent slighter sensitiveness, had accumulated a sufficiently high concentration of circulating antibodies to make possible a passive transfer to normal guinea pigs, resulting in acute death following the injection of the antigen. In order to provide a more concrete explanation of the reduced sensitiveness observed in blocked guinea pigs, it was necessary to carry out a quantitative titration by passive transfer, sufficiently accurate to detect any difference in the titer of anaphylactic antibodies in blocked and in normal animals. Simultaneously, it seemed of interest to study the precipitin titer in such serums in comparison with their anaphylactic power. As these experiments called for greater amounts of serum, rabbits were used for the production of the antiserum and guinea pigs for the passive transfer.

Two rabbits (Nos. 1 and 2) were each given three intravenous ink injections of 5 cc. of a 1:4 dilution at 2 day intervals, and then four intravenous injections of normal horse serum (1 cc., 2 cc., 3 cc., 3 cc.) at 3 day intervals, each time preceded by another ink injection of the same dose. Two rabbits (Nos. 3 and 4) were treated on the same schedule with ink injections of 5 cc. of a 1:50 dilution and sensitized in the same manner. Two control rabbits (Nos. 5 and 6) were given 5 cc. of salt solution intravenously, whenever the other four animals received ink injections, and were sensitized with the same doses. All the animals in each case weighed from 1800 to 2200 gm. Blood was taken before treatment, 2 and 4 days after the last injection, and, on the 6th day, all the animals were bled to death. The precipitin titer of the different serums was not determined by the Uhlenhuth method, but by di uting the antiserum and keeping the antigen constant. The results obtained are shown in Table V.

TABLE V.

Determination of Precipitin Titer of Rabbit Antiserums.

Rabbit No.	Trial bleeding. Date.	Trial bleeding. Time since last antigen injection.	Dilutions. 1:5	1:10	1:20	1:40	1:80	1:100	1:160	1:200	1:320	1:640	1:1000
		days											
1*	July 28		±	−	−								
	Aug. 14	2		+	+	−	−	−	−	−			
	" 16	4		+	+	−	−	−	−	−			
	" 18	6		+	+	+	−	−	−	−	−	−	−
2*	July 28		+	−	−								
	Aug. 14	2		+	+	−	−	−	−	−			
	" 16	4		+	+	+	−	−	−	−			
	" 18	6		+	+	+	+	+	+	−	−	−	−
3†	July 28		±	−	−								
	Aug. 14	2		+	+	+	+	+	+	−			
	" 16	4		+	+	+	+	+	+	+	+		
	" 18	6		+	+	+	+	+	+	+	+	+	−
4†	July 28		±	−	−								
	Aug. 14	2		+	+	+	+	−	−	−			
	" 16	4		+	+	+	+	+	+	−			
	" 18	6		+	+	+	+	+	+	+	−	−	−
5‡	July 28		±	−	−								
	Aug. 14	2		+	+	+	+	−	−	−			
	" 16	4		+	+	+	+	+	+	−			
	" 18	6		+	+	+	+	+	+	+	+	−	−
6‡	July 28		−	−	−								
	Aug. 14	2		+	+	+	−	−	−	−			
	" 16	4		+	+	+	+	−	−	−			
	" 18	6		+	+	+	+	+	−	−	−	−	−

* Received large doses of ink.

† Received small doses of ink.

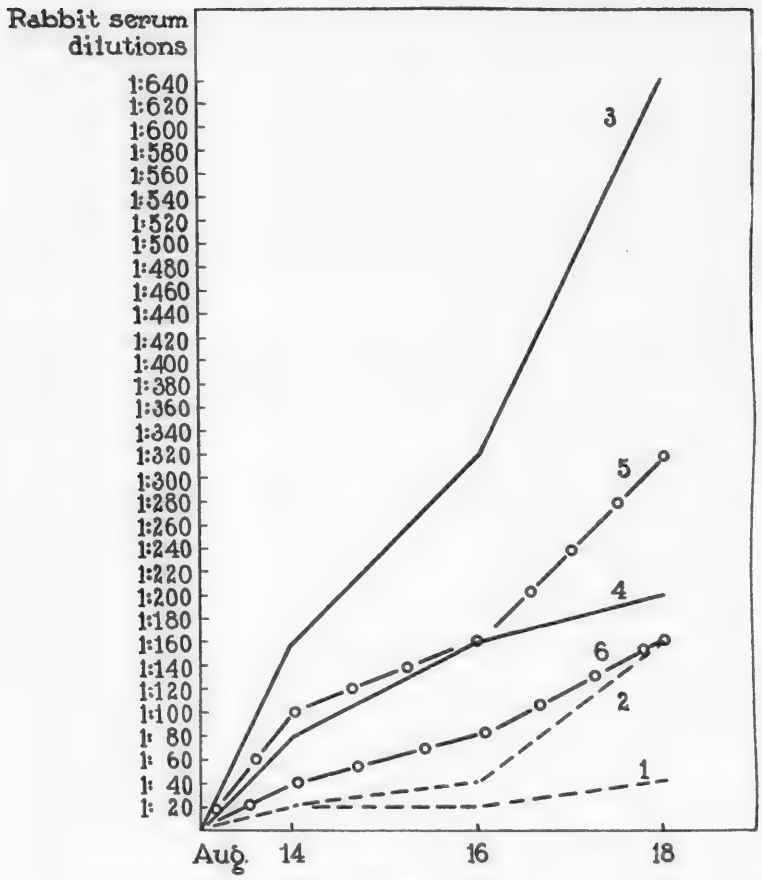
‡ Received salt solution.

Technique:

Rabbit serum dilutions 0.5 cc.

Normal horse serum undiluted (constant) 0.5 cc.

Incubation 1 hour at 37°C.



TEXT-FIG. 1.

——— Animals injected with small doses of ink.

— — — Animals injected with large doses of ink.

—o—o— Control animals injected with physiological salt solution.

Comparison of the precipitin titer of antiserums in rabbits injected with small and large doses of India ink.

Technique:

Titer determined after 1 hour's incubation at 37°C.

Rabbit serum dilutions: 0.5 cc.

Normal horse serum undiluted: 0.5 cc. (constant).

It appears from this table and also from the accompanying graph that the serum from the two blocked animals had the lowest precipitin titer, though eventually one serum reached the same height as one control serum. On the other hand, the serum of one animal which had received the smaller ink doses showed throughout the highest titer of precipitins, while the serum from the other animal treated in the same way had about the same titer as the other control serum.

All six serums (final bleedings) were tested for their anaphylactic power in guinea pigs according to the method outlined by Doerr and Russ (17).

1 cc. of each serum was injected intraperitoneally into each of four guinea pigs, making a total of twenty-four guinea pigs, and 24 hours later all the guinea pigs were injected intravenously with graduated amounts of normal horse serum, four different amounts being used for each particular serum. The results of this test are shown in Table VI.

It appears from this table that the serum from one blocked animal (No. 1) had a slightly lower anaphylactic titer than that of two normal control animals, a dose of 0.05 cc. of normal horse serum being followed by death after only 2 hours, and the doses of 0.02 and 0.01 cc. being tolerated without any symptoms. The anaphylactic titer of the serum of the other blocked animal did not differ appreciably from that of the controls. The anaphylactic titer of the serum of two animals treated with the smaller doses of ink was not different from the titer of the control animals. Comparison with the precipitin titer shows that in one instance (blocked rabbit No. 1 and controls Nos. 5 and 6) a lower precipitin titer ran parallel with a slightly lower anaphylactic titer, while in another instance of a higher precipitin titer (rabbit No. 3 and two controls) no such parallelism was observed.

The results obtained in this experiment were essentially negative. They by no means furnish proof that the production of circulating anaphylactic antibody in the blocked sensitized rabbit is lower than that of the normal sensitized rabbit. It seems justifiable, however, to conclude that the formation of precipitins in blocked sensitized rabbits is temporarily inhibited as compared with normal sensitized control animals. As regards the effect of smaller doses of ink, it would seem that in one case they caused a stimulation in the production of

TABLE VI.

Titration of Anaphylactic Power of Rabbit Antiserums by Passive Transfer to Guinea Pigs.

Guinea pig No.	Sensitizing injection.*			Reinjections of normal horse serum No. 140.†		Results.
	Serum from Rabbit No.	Date.	Dose.	Date.	Dose.	
			cc.		*cc.*	
48	1‡	Aug. 21	1	Aug. 22	0.1	Died in 4 min.
49	1	" 21	1	" 22	0.05	Very severe symptoms. Died in 2 hrs.
50	1	" 21	1	" 22	0.02	No symptoms. Survived.
51	1	" 21	1	" 22	0.01	" " "
52	2‡	" 21	1	" 22	0.1	Died in 4 min.
53	2	" 21	1	" 22	0.05	" " 10 "
54	2	" 21	1	" 22	0.02	No symptoms. Survived.
55	2	" 21	1	" 22	0.01	Slight symptoms. Survived.
56	3§	" 21	1	" 22	0.1	Died in 4 min.
57	3	" 21	1	" 22	0.05	" " 4 "
58	3	" 21	1	" 22	0.02	Moderate symptoms. Survived.
59	3	" 21	1	" 22	0.01	No symptoms. Survived.
60	4§	" 22	1	" 23	0.1	Died in 8 min.
61	4	" 22	1	" 23	0.05	" " 10 "
62	4	" 22	1	" 23	0.02	Slight symptoms. Survived.
63	4	" 22	1	" 23	0.01	No symptoms. Survived.
64	5‖	" 22	1	" 23	0.1	Died in 3 min.
65	5	" 22	1	" 23	0.05	" " 4 "
66	5	" 22	1	" 23	0.02	Severe symptoms. Survived.
67	5	" 22	1	" 23	0.01	Moderate symptoms. Survived.
68	6‖	" 22	1	" 23	0.1	Died in 3 min.
69	6	" 22	1	" 23	0.05	" " 4 "
70	6	" 22	1	" 23	0.02	Slight symptoms. Survived.
71	6	" 22	1	" 23	0.01	No symptoms. Survived.

Autopsy findings in each case were typical.

* All sensitizing injections were intraperitoneal.

† All reinjections were intravenous.

‡ Received large doses of ink.

§ Received small doses of ink.

‖ Received salt solution.

precipitins, while the anaphylactic power of the serum of rabbits treated with these smaller doses was similar to that of the controls.

C. Experiments on the Effect of Blockade before Passive Sensitization on the Occurrence of Anaphylactic Shock in Guinea Pigs.

While the diminution of sensitiveness observed in blocked, actively sensitized guinea pigs might be considered analogous to the interference of blockade in processes of active immunization, no such

TABLE VII.

The Production of Passive Anaphylaxis in the Blocked Guinea Pig.

Guinea pig No.	Blocking injections of ink 1:5 dilution on Aug. 27.*	Sensitizing injection of rabbit serum No. 5 on Aug. 27.†	Reinjections of normal horse serum No. 140 on Aug. 28.*	Results.
	Dose.	Dose.	Dose.	
	cc.	*cc.*	*cc.*	
72	1.5	1	0.05	Died in 4 min.
73	1.5	1	0.05	" " 6 "
74	1.5	1	0.02	Moderate symptoms. Survived.
75	1.5	1	0.02	Severe " "
76		1	0.05	Died in 4 min.
77		1	0.05	" " 5 "
78		1	0.02	Severe symptoms. Survived.
79		1	0.02	Slight " "

Autopsy findings in each case were typical.

* Blocking injections and reinjections were intravenous.

† Sensitizing injections were intraperitoneal.

mechanism could be thought of in passive anaphylaxis. Blocking experiments before passive sensitization were done with a view to obtaining data concerning the extent to which cellular changes of the organs of the blocked animals, effected by the absorption of ink, were involved in the reduction of sensitiveness.

Four guinea pigs of from 230 to 280 gm. in weight were given one intravenous injection of 1.5 cc. ink, diluted 1:5, and were passively sensitized 1 day later, together with four normal controls, by intraperitoneal injection of 1 cc. of rabbit antiserum No. 5. All eight guinea pigs were reinjected 24 hours later, four with a

lethal and four with a sublethal dose of normal horse serum. The results obtained are given in Table VII.

No difference in sensitiveness between the two groups was observed. It would seem justifiable to conclude from this experiment that the occurrence of anaphylactic shock in passively sensitized guinea pigs is not interfered with by giving one blocking injection of India ink before this sensitization.

DISCUSSION.

The observations here recorded agree in principle with the experience of many other authors, that any active immunization process in blocked animals is inhibited in direct proportion to the extent of elimination of the reticulo-endothelial system. This is especially true of the formation of precipitins in the blocked animal as recently reported by Gay and Clark (18). The quick regeneration of the blocked system obviously makes it impossible to obtain more than relative results with the present methods of blockade. It is doubtful whether any procedure can be developed which would accomplish more without endangering vital physiological functions of life, except possibly a combination of blocking with splenectomy.

We feel inclined, in view of the positive results we have obtained before, to ascribe to the reticulo-endothelial system singular importance in the production of antibodies. This opinion is considerably strengthened by the observations made during the course of this study. The precipitin titer of the serum of blocked, actively sensitized rabbits was definitely lower than that of normal sensitized controls, while smaller doses of ink in one case were followed by a higher precipitin titer than that seen in controls. Our experiments demonstrate further that the anaphylactic shock in the majority of guinea pigs which received blocking injections of ink before active sensitization, assumed a definitely milder and more protracted form and that occasionally a blocked, actively sensitized animal will survive even a dose of antigen fatal to the controls. Our experiments do not intimate a convincing explanation for this reduced sensitiveness since a titration by means of passive anaphylaxis in guinea pigs showed the blood serum of blocked, sensitized rabbits to contain approximately the same amount

of anaphylactic antibody as that of the controls. The fact that blocking before passive sensitization did not interfere with the occurrence of the anaphylactic shock in any way seems to indicate that, if the animal plays no active part in the production of the anaphylactic antibody, the mere presence of ink, under the conditions of our experiments, does not affect the animal's susceptibility to the anaphylactic reaction as such. Finally, it is of interest to note that there was no complete parallelism between the precipitin titer and the anaphylactic power of the serum of several ink-treated sensitized rabbits. While the lower precipitin titer in a blocked animal in one case corresponded with a slightly lower anaphylactic power, this was not true with the higher precipitin titer and the anaphylactic power of the serum of one rabbit which had received the smaller doses of ink.

SUMMARY AND CONCLUSIONS.

1. Guinea pigs injected intravenously with massive doses of India ink before active sensitization exhibited occasionally a more or less marked, decreased sensitiveness to the reinjection of the antigen.

2. The serum of rabbits which had received massive doses of India ink before the sensitizing injections, showed approximately the same titer of anaphylactic antibodies as that of sensitized normal control animals, as demonstrated by the degree of passive sensitization induced in guinea pigs.

3. The precipitin titer of sensitized rabbits blocked with massive doses of India ink was somewhat lower than that of sensitized normal controls. In one instance, the intravenous injection of smaller doses of India ink was followed by a higher precipitin titer. No uniform relation was found between the height of the precipitin titer and the anaphylactic power of the antiserums.

4. One blocking injection of India ink given to guinea pigs before passive sensitization did not interfere with the occurrence of anaphylactic shock nor alter its nature.

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